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Cellular Expression of α-Gustducin and the A Blood Group Antigen in Rat Fungiform Taste Buds Cross-Reinnervated by the IXth Nerve

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ABSTRACT

Although taste buds are trophically dependent on their innervation, cross-reinnervation experiments have shown that their gustatory sensitivities are determined by the local epithelium. Both the gustatory G-protein, α-gustducin, and the cell-surface carbohydrate, the A blood group antigen, are expressed by significantly fewer fungiform than vallate taste cells in the rat. In these experiments, one side of the anterior portion of the tongue was cross-reinnervated by the IXth nerve in order to determine whether the molecular expression of taste bud cells is determined by the epithelium from which they arise or by the nerve on which they are trophically dependent. The proximal portion of the IXth nerve was anastomosed to the distal portion of the chorda tympani (CT) nerve using fibrin glue (IX-CT rats). Control animals had the CT cut and reanastomosed using the same technique (CT-CT rats), or had the CT avulsed from the bulla and resected to prevent regeneration (CTX rats). The animals survived for 12 weeks postoperatively, and the tongues were removed, stained with methylene blue, and the fungiform taste pores counted on both sides. Tissue from the anterior 5 mm of the tongue was cut into 50-μm sections, which were incubated with antibodies against α-gustducin and the human blood group A antigen. In both CT-CT and IX-CT rats, there was regeneration of fungiform taste buds, although in both groups there were significantly fewer taste buds on the operated side of the tongue. The normal vallate papilla had a mean of 8.37 α-gustducin-expressing cells and 5.22 A-expressing cells per taste bud, whereas the fungiform papillae contained 3.06 and 0.23 cells per taste bud, respectively. In both CT-CT and IX-CT rats there was a normal number of cells expressing α-gustducin or the A antigen in regenerated taste buds; in the CTX animals there was a significant decrease in the expression of these markers. These results demonstrate that the molecular phenotype of taste bud cells is determined by the local epithelium from which they arise and not by properties of the innervating nerve. J. Comp. Neurol. 409:118–130, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: taste bud cells; gustation; chorda tympani nerve; glossopharyngeal nerve

Mammalian taste receptor cells are found within several distinct populations of taste buds (Miller et al., 1978; Miller and Smith, 1984; Belecky and Smith, 1990). Taste buds in the fungiform papillae on the anterior two-thirds of the tongue are innervated by the chorda tympani (CT) branch of the VIIth nerve, and those in the vallate and foliate papillae on the posterior one-third of the tongue are innervated by the glossopharyngeal (IXth) nerve (Whiteside, 1927; Oakley, 1970); some of the more rostral foliate papillae are innervated by the CT. The differentiation of taste receptor cells within the gustatory epithelium and the maintenance of taste bud morphology are dependent upon the innervation of the epithelium by gustatory nerve fibers. Transection of the glossopharyngeal nerve results

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in degeneration of the vallate taste buds within 7 days (Guth, 1957), and the taste buds reappear following regeneration of the peripheral nerve fibers (Iwayama and Nada, 1969; Hosley et al., 1987). Similar effects are seen in fungiform taste buds (Cheal and Oakley, 1977), although there is some evidence that remnant taste buds may remain after CT nerve transection (Whitehead et al., 1987; Hård af Segerstad et al., 1989). The trophic influence of the nerve appears to require axoplasmic transport (Sloan et al., 1983). Only taste bud-bearing epithelium and chemosensory nerves (including the vagus and, specifically, the carotid sinus nerve) are competent to support the formation and maintenance of taste buds (Guth, 1958; Zalewski, 1970; Oakley, 1974, 1985; Sanchez et al., 1978; Zalewski, 1981; Dinger et al., 1984). Thus, it is generally believed that factors specific to gustatory nerves are important for their trophic interaction with taste cells.

Electrophysiological studies of the responsiveness of gustatory nerve fibers in rodents have shown that the sensitivities of these various taste bud populations differ considerably (Smith and Frank, 1993). In the rat, for example, the fibers of the CT nerve are much more responsive to NaCl than those of the IXth nerve, whereas the IXth nerve fibers are more responsive to quinine (Pfaffmann, 1955; Frank et al., 1983; Frank, 1991). To test whether the difference in sensitivity between the fungiform and vallate taste buds was trophically dependent upon their nerve supplies, Oakley (1967) recorded the activity of these nerves following cross-reinnervation of the taste buds. That is, the anterior tongue was reinnervated by the IXth nerve or the posterior tongue was reinnervated by the CT nerve following cross-anastomosis of the proximal portion of one nerve to the distal portion of the other. Regardless of whether the fungiform papillae were innervated by the CT nerve or the IXth nerve, NaCl was a much more effective stimulus than quinine, whereas quinine was a more effective stimulus for the vallate taste buds, even when they were innervated by the CT nerve. Similar results were seen following cross-regeneration of the CT and the greater superficial petrosal (GSP) nerves in the rat, which differ in their sucrose sensitivity (Nejad and Beidler, 1987). These data suggest that, even though the taste cells themselves are trophically dependent on their innervation, the receptor phenotypes expressed in the taste cells are determined by the epithelium.

Taste cells express several molecular markers, including the neural cell adhesion molecule (Nolte and Martini, 1992; Nelson and Finger, 1993; Smith et al., 1993), neuron-specific enolase (Yoshie et al., 1989; Montavon and Lindstrand, 1991), keratin-19 (Oakley et al., 1993), the G-protein α-gustducin (McLaughlin et al., 1992; Boughter et al., 1997), and a number of cell-surface carbohydrates, including several blood group antigens (Smith et al., 1994b). There are differences in the distribution of these markers between the various populations of taste buds, especially between fungiform and vallate taste buds. For example, the number of cells in each taste bud expressing α-gustducin is three times greater in the rat vallate than in the fungiform papillae (Boughter et al., 1997). This difference likely reflects differences in sensitivity to sweet and/or bitter stimuli between the anterior and posterior tongue. Cell-surface carbohydrates also appear to differ in their distribution between these taste bud populations. The A blood group antigen, for example, is considerably more prominent in vallate taste bud cells than in those of the fungiform taste buds (Smith et al., 1994b), although this difference has not been rigorously quantified. The expression of these and other molecules by taste cells appears to be largely dependent upon innervation, because nerve transection results in elimination of immunoreactivity (Smith et al., 1993, 1994a, b), except where remnant taste buds occur in some fungiform papillae (Whitehead et al., 1998).

In order to test whether the trophic influence of gustatory innervation determines the molecular expression of taste cells, we examined the expression of α-gustducin and the A blood group antigen in rat fungiform taste buds following reinnervation by the CT nerve or cross-reinnervation by the IXth nerve. Both of these markers occur in significantly greater numbers of vallate taste cells than fungiform (Smith et al., 1994b; Boughter et al., 1997), providing a clear opportunity to observe an increase in fungiform taste cell expression following innervation by the IXth nerve.

MATERIALS AND METHODS

Animals and surgery

Data were obtained from 21 young adult male Sprague-Dawley rats. Six of these animals (IX-CT group) underwent a successful unilateral cross-anastomosis of the proximal portion of the left glossopharyngeal (IX) nerve to the distal portion of the left chorda tympani (CT) nerve, as described below. Another six (CT-CT group) had their left CT nerve transected and then reanastomosed following the same procedure as the cross-reinnervated group. An additional three animals (CTX group) had their CT nerve avulsed from the bulla and resected to prevent regeneration. Six unoperated rats (Intact group) were used to assess the distribution of fungiform papillae and taste pores on the two sides of the tongue. These unoperated animals were not included in the immunohistochemical study, in which the intact right side of the tongue served as a control for each animal. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

For the cross-anastomosis of IX to CT, 18 rats were anesthetized with chloral hydrate (40 mg/kg i.p.). A 4-cm midline excision revealed the left digastric muscle, which was retracted laterally to expose the hyoid bone lying medial to the hypoglossal nerve. The hyoid was dissected free from the strap musculature and removed unilaterally. Dissection was then carried medial and deep to the external carotid artery to expose the IXth nerve. After adequate rostral dissection, the IXth nerve was transected and the proximal portion brought to the caudal aspect of the dissection. The medial pterygoid muscle was identified deep and slightly medial to the external carotid, rostral to the point where IX was cut. The medial pterygoid was retracted rostrally while dissection proceeded down to the posterior facial vein (PFV), which lies immediately rostral to the bulla. The vein from the pterygoid plexus (VPP) was identified entering the PFV rostrally. The CT was seen running almost parallel to this vein and crossing under it. The CT was then carefully dissected free from both the lateral pterygoid muscle and the VPP and avulsed from the bulla at the petrotympanic fissure, providing an adequate length for anastomosis with IX. The CT was brought out to the area of the IXth nerve dissection. After

approximation of IX and CT on a piece of Gelfilm, powdered bovine thrombin (~ 500 U.S. units) was sprinkled onto the cut ends of the two nerves, followed by 0.1–0.2 ml of thawed human pooled (Type A and Type AB) cryoprecipitate, and 5 minutes allowed for fibrin formation, which was readily visualized (Matras, 1985; Lerner and Binur, 1990). The wound was closed and the animal allowed to survive for at least 12 weeks. Just prior to perfusion and tissue extraction, the animals were anesthetized and the site of anastomosis was exposed to ensure that the ends of the IXth nerve and CT were still in contact. Of these 18 animals, 6 showed successful reinnervation (IX-CT group), defined: (1) by an intact IX-CT anastomosis, and (2) by the number of fungiform taste pores on the first 5 mm of the tongue being greater than 50% of the number on the unoperated side (see below). Twelve animals had less than 28% regeneration of taste pores (and in some cases a disruption of the IX-CT anastomosis) and were not included in the quantification of α-gustducin and A blood group antigen expression. An additional nine rats were included in the CT-CT group and the CTX group, as described above.

Tongues of all animals were removed, stained with methylene blue, and the fungiform papillae and taste pores were counted on both sides. Tissue from the anterior 5 mm of the tongue and the region of the vallate papilla on the posterior tongue (groups IX-CT, CT-CT, and CTX) was cut into 50-μm sections, and every second section was reacted with monoclonal antibodies against α-gustducin and the human blood group A antigen.

Tissue preparation and sectioning

Rats were perfused through the left ventricle with room-temperature phosphate-buffered saline (PBS) followed by ice-cold (4°C) fixative containing 4% formaldehyde and 2% sucrose in 0.1 M phosphate buffer (PB); all solutions were pH 7.3–7.4. The tongue was removed immediately after fixation, the posterior portion containing the vallate papilla was excised, and the entire remaining anterior portion of the tongue was cut in half down the midline. Each tongue half and the vallate block was postfixed for 4 hours, during which the fixative was changed three times. After postfixation, the tongue portions were placed in 30% sucrose in 0.1 M PB overnight.

The next day, the anterior tongue halves were removed from the sucrose, stained with methylene blue, and the fungiform papillae and taste pores on the entire anterior tongue were counted using a dissecting microscope (50×; Miller and Bartoshuk, 1991; St. John et al., 1995). In order to facilitate these counts and to map the distribution of taste pores, a transparent 6 × 11 grid of 2.5-mm squares was laid over the tongue and the locations of papillae and pores was mapped onto a corresponding grid overlaying a schematic diagram of the tongue (see Fig. 1). Animals were included in the IX-CT and CT-CT groups if the number of pores on the most anterior 5 mm of the left (surgical) side of the tongue was at least 50% of the number on the right, indicating a successful reinnervation of the fungiform taste buds.

Following quantification of taste pores, the anterior 5 mm of each tongue half and the tissue containing the vallate papilla were embedded in Tissue Tek, frozen in −70°C isopentane, and cut coronally on a cryostat into 50-μm sections. Care was taken to maintain the identity of the left and right halves of the tongue.

Antibodies and immunohistochemistry

We used an affinity-purified rabbit IgG polyclonal antibody (Gα gust [I-20]; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against a peptide corresponding to amino acids 93–112 mapping within a highly divergent domain of rat α-gustducin. Gα gust (I-20) reacts specifically with α-gustducin of mouse, rat, and human cell origin as shown by Western blotting and immunohistochemistry; it lacks cross-reactivity with other Gα subunits, including rod or cone α-transducin (Santa Cruz Biotechnology, Inc.). Immunoreactivity to Gα gust (I-20) was not seen in cryostat sections prepared from rat or hamster retina (Boughter et al., 1997). There are significantly more cells expressing α-gustducin in rat vallate and foliate taste buds than in fungiform taste buds (Boughter et al., 1997).

A monoclonal antibody against the human blood group A antigen (clone 81FR2.2) was obtained from the Dako Corporation (Carpinteria,CA). The anti-A antibody, an IgM against the synthetic trisaccharide:

$$\left.\begin{matrix}\text{Fuc}\alpha 1 \rightarrow 2 \\ \text{GalNAc}\alpha 1 \rightarrow 3\end{matrix}\right\rangle \text{GalB}-$$

recognizes subtypes A1 and A2 on cell membranes of epithelium, endothelium, and erythrocytes. The A blood group antigen has been previously described on taste bud cells of the rat, where it was shown to be more prevalent in vallate and foliate taste buds than in fungiform (Smith et al., 1994b).

Fluorescent secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). As a secondary to the α-gustducin reaction, we used Cy™3-conjugated affinity-purified goat anti-rabbit IgG (H + L) fluorescent antibody and for the A blood group antigen, Cy™2-conjugated affinity-purified goat anti-mouse IgG + IgM (H + L) fluorescent antibody.

Cryostat sections (50 μm) of the fungiform and vallate taste buds were preincubated in 0.3% Triton X-100, 0.1% bovine serum albumen (BSA), and 10% normal goat serum in PBS for 1 hour at room temperature. Sections were then incubated with the primary antibodies against α-gustducin diluted 1:75 and the A blood group antigen diluted 1:50 in freshly prepared preincubation solution for 2–3 hours at room temperature. Immunoreacted sections were rinsed in PBS, then incubated with a mixture of the secondary antibodies diluted 1:100 in PBS for 1 hour in a cold room (4°C). Sections were then rinsed in PBS and stored overnight in PBS in the cold room. Finally, tissue sections were mounted onto glass slides and coverslipped using ProLong™ Antifade media (Molecular Probes, Inc., Eugene, OR). Nonimmune control sections processed in the same fashion, but without the primary antibody, were always negative. Previously, we demonstrated that preincubation with the blocking peptide would prevent immunoreactivity to the α-gustducin antibody in rat vallate taste cells (Boughter et al., 1997).

Cell quantification

Immunoreactive taste bud cells were observed and photographed with a 40×/1.0 n.a. or 60×/1.4 n.a. oil-immersion lens on an Olympus Fluoview confocal dual-laser scanning microscope (CLSM). Using the CLSM, a series of optical sections was acquired at 1 or 2 μm

intervals and then viewed successively to count cells. To examine the labeling of cells with the two antibodies, two stacks of optical sections were taken in quick succession starting at the same specimen depth. One stack was obtained from the Cy™3 channel (α-gustducin) and the other stack from the Cy™2 channel (A antigen). Corresponding images taken at the same depth in each stack were rendered in red and green and superimposed to obtain the final colored image. Confocal images were stored on Zip disks (Iomega Corp., Roy, UT). Photomicrographs were prepared from these stored image using Adobe Photoshop (v. 4.0; Adobe Systems, Inc., San Jose, CA). Brightness, contrast, and color balance were adjusted on the CLSM or with Photoshop prior to printing on a Fujix Pictrography 3000 dye-sublimation printer (Fuji Photo Film U.S.A., Elmsford, NY).

The number of immunoreactive cells in every fungiform taste bud in every other 50-μm section of the anterior 5 mm of both sides of the tongue was determined for all animals in the CT-CT, IX-CT, and CTX groups. In order for a cell to be included in the counts, the section had to include the cell nucleus and/or an apical process converging toward the taste pore (see Boughter et al., 1997). Basal fragments of cells were not counted. Because the number of cells expressing these antigens in fungiform taste buds is relatively low, and because sections through fungiform papillae do not necessarily contain taste buds, the presence or absence of taste buds in every section was confirmed by observing the sections under Hoffman illumination. This step allowed us to confirm the presence of taste buds containing no immunolabeled cells. The mean numbers of labeled cells were then based on the total number of taste buds in the sections, including those containing zero labeled cells. Cells were counted in a single section through the vallate papillae of each of three of the CT-CT animals in order to determine the number of cells present in these taste buds. The innervation of the vallate papilla by the IXth nerve was not interrupted in this group. In these vallate sections, cells in all taste buds were counted except those that were superimposed in the section.

RESULTS

Regeneration of taste pores on the anterior tongue

The distributions of fungiform papillae and taste pores on the anterior tongue of individual animals in each group are depicted in Figure 1.

Taste pores are indicated by small filled circles and fungiform papillae without visible pores by open circles. Different regions on the anterior tongue are indicated by the superimposed grid, which overall is the same size as the counting grid used to quantify the distribution of taste pores and papillae. In order to statistically evaluate the distribution of taste buds, the 2.5-mm squares of the counting grid were combined so that each of the squares (7.5 mm per side) in Figure 1 represents nine of the smaller squares, dividing the CT field on each side into thirds. The anterior one-third of this area corresponds approximately to the first 5 mm of the tongue, which was sectioned for the immunohistochemical studies. The lateral dashed lines in these diagrams indicate lateral and ventral portions of the tongue surface; numerous fungiform papillae and taste pores were seen on the ventral surface of the anterior tip of the tongue. As previously shown for the rat (Miller and Bartoshuk, 1991; St. John et al., 1995), almost two-thirds of the fungiform taste buds were contained in the most anterior 5 mm of the tongue (see Fig. 1A or the control sides of B, C, or D). Transection of the CT nerve (Fig. 1B) resulted in near total loss of taste pores and a reduction in the number of fungiform papillae. Remnant taste pores occurred almost exclusively in the most anterior portion of the CT field. Either reanastomosis (CT-CT; Fig. 1C) or cross-anastomosis (IX-CT; Fig. 1D) resulted in a substantial number of taste pores on the operated side of the tongue, although in both surgical animals there were more fungiform papillae without taste pores than in the intact rat or on the unoperated side of the surgical rats.

The data from these papilla and taste pore counts are summarized in Figure 2, which shows the means (+S.E.M.) for each square of the grids depicted in Figure 1 for all of the animals in each group. For the Intact group (Fig. 1A), a repeated measures two-way analysis of variance (ANOVA) demonstrated a significant difference among tongue regions ($F[2,10] = 105.7$, $P < 0.001$), confirming the observation that fungiform taste buds are not evenly distributed across the anterior tongue. There was no difference between the two sides ($F[1,5] = 0.068$, $P = 0.805$). When the CT nerve was severed and then glued back together (CT-CT group; Fig. 1B), there was a significant anterior-posterior difference in the distribution of taste buds ($F[2,10] = 115.4$, $P < 0.001$) and a significant difference between the intact and operated sides ($F[1,5] = 12.19$, $P = 0.017$), suggesting that the CT-CT reanastomosis did not result in complete regeneration of the fungiform taste buds. Nevertheless, on the most anterior portion of the CT field, the taste pores on the operated side regenerated to 83.3% of the number on the unoperated side. There was no significant interaction between tongue region and side ($F[2,10] = 2.48$, $P = 0.133$).

For the six animals included in the IX-CT group (Fig. 1C), there was a significant anterior–posterior difference in taste pore distribution on the anterior tongue ($F[2,10] = 210.05$, $P < 0.001$) and a significant difference between the two sides ($F[1,5] = 24.97$, $P = 0.004$). In addition, there was a significant interaction between region and side ($F[2,10] = 5.36$, $P = 0.026$). Post-hoc comparisons demonstrated that the difference between the two sides was significant for anterior ($F[1,5] = 12.34$, $P = 0.017$), middle ($F[1,5] = 35.59$, $P = 0.002$), and posterior ($F[1,5] = 15.63$, $P = 0.011$) regions (one-way repeated measures ANOVA). In spite of the failure of these animals to regenerate their normal complement of fungiform taste buds, the degree of regeneration was comparable to that seen in the CT-CT group; the number of pores on the most anterior part of the tongue on the cross-regenerated side was 75.1% of that on the unoperated side. In contrast, in the CTX group (Fig. 1D) the number of taste pores on the entire cut side was only 12.4% of that on the uncut side. For the CTX group, there was a significant anterior- posterior difference in taste pore distribution ($F[2,4] = 41.73$, $P = 0.002$) and a significant difference between the two sides ($F[1,2] = 316.11$, $P = 0.003$). In addition, there was a significant interaction between region and side ($F[2,4] = 285.86$, $P < 0.001$). Post-hoc comparisons demonstrated that the difference between the two sides was significant for the anterior ($F[1,2] = 2689.92$, $P < 0.001$) and middle ($F[1,2] = 25.33$, $P = 0.037$) regions (one-way repeated measures ANOVA).

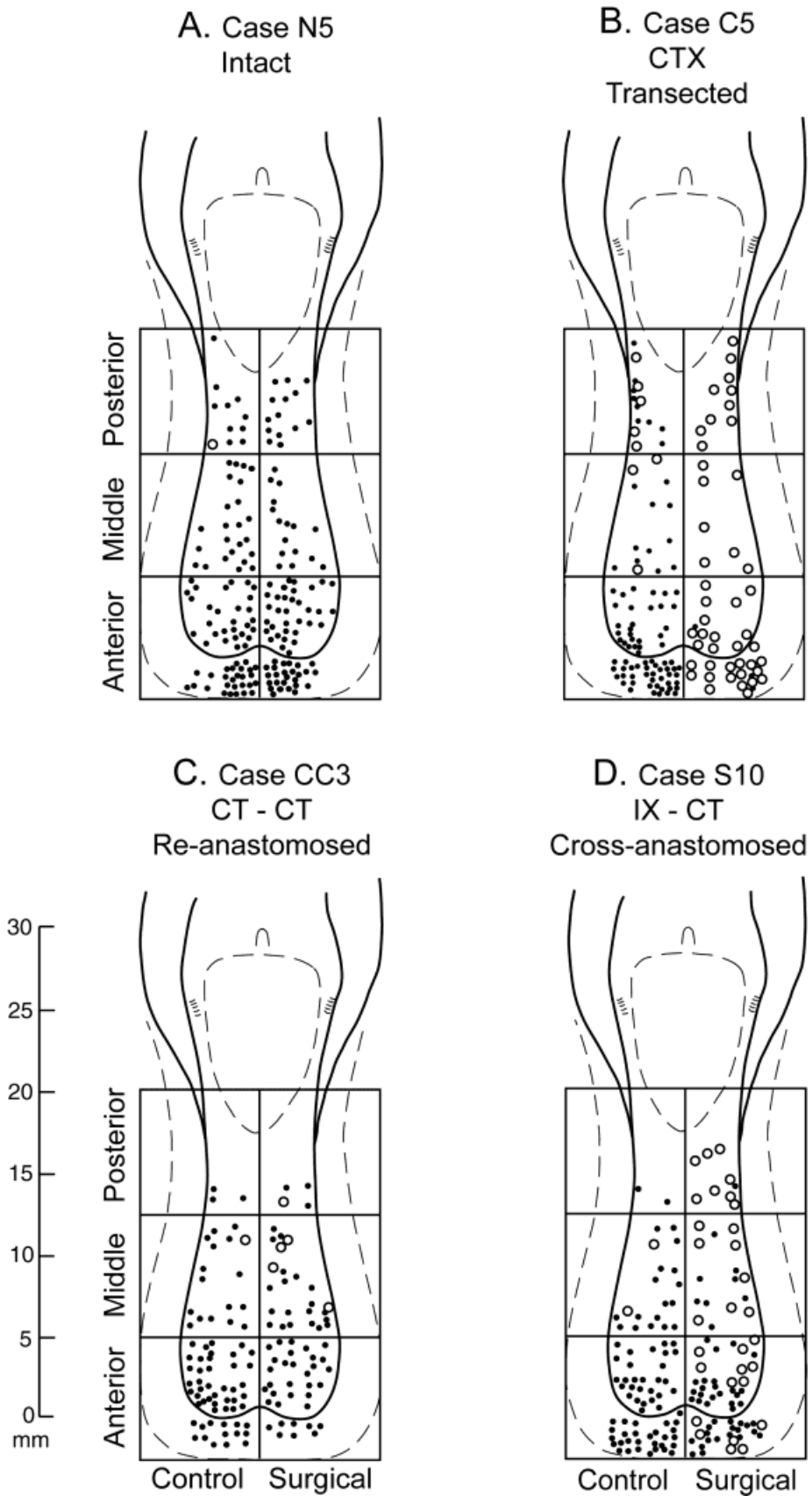

Fig. 1. Schematic diagrams of the rat tongue showing the distribution of fungiform taste pores (solid circles) and papillae without pores (open circles) from four individual rats from each of four surgical groups. Papillae or pores shown outside the solid border of the tongue were located on the anterior ventral epithelium. Superimposed over each diagram is a 15 × 22.5 mm grid, delineating different regions and sides of the anterior tongue. **A:** Tongue of an intact rat. **B:** Tongue of a rat with a left chorda tympani (CT) nerve transection (CTX). **C:** Tongue of a rat in which the left CT nerve was transected and then reanastomosed. **D:** Tongue of a rat in which the distal portion of left CT nerve was cross-anastomosed to the proximal portion of the left glossopharyngeal (IXth) nerve. Survival times of the surgical animals were greater than 12 weeks.

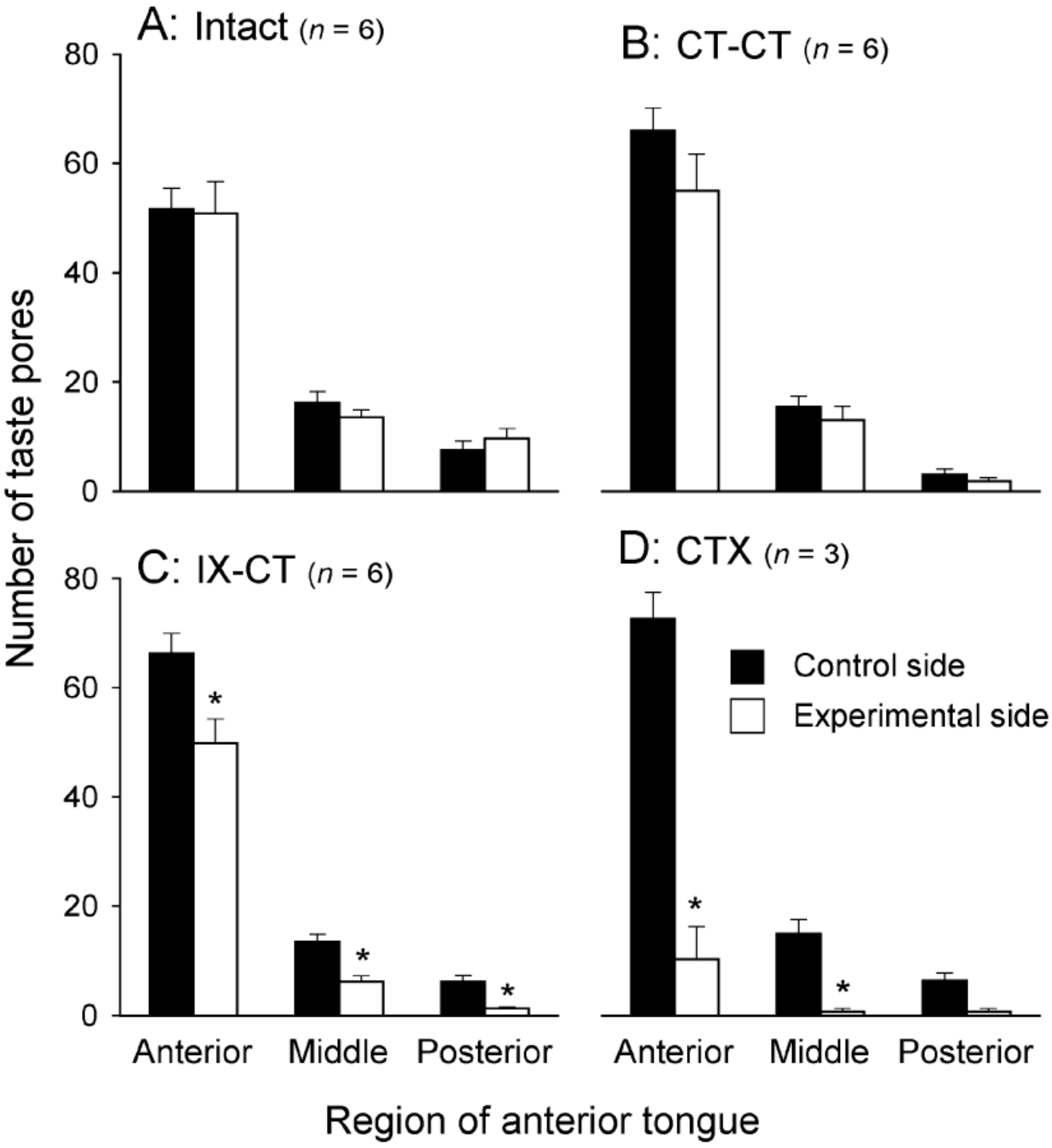

Fig. 2. Mean numbers (+S.E.M.) of taste pores in each region of both sides of the anterior tongue in intact rats (**A**), chorda tympani (CT)-CT reanastomosed rats (**B**), glossopharyngeal nerve (IX)-CT cross-anastomosed rats (**C**), and CT transected (CTX) rats (**D**). The numbers of animals in each group are indicated. Asterisks show significant differences that were demonstrated by post-hoc tests; there was a significant difference between the two sides in the CT-CT group, but no significant interaction between region and side (see text).

In addition to reducing the number of taste pores, cutting the CT nerve reduced the number of fungiform papillae. The mean number of fungiform papillae on the entire anterior tongue of the animals in each group are shown in Table 1. A two-way repeated measures ANOVA demonstrated no difference between the groups ($F[3,17] = 0.46$, $P = 0.714$), but a significant difference between the two sides of the tongue ($F[1,17] = 51.03$, $P < 0.001$) and a significant interaction between group and side ($F[1,17] = 11.49$, $P < 0.001$). Post-hoc analyses (one-way repeated

TABLE 1. Counts[1] of the Number of Fungiform Papillae in Each Group

Group/side	Number of rats	Survival time (days)	Number of fungiform papillae
Intact/right	6	n.a.[5]	76.83 ± 6.94
Intact/left			75.83 ± 7.94
CT-CT[2]/right	6	88.33 ± 2.03	88.83 ± 6.10
CT-CT/left			79.17 ± 6.80
IX[3]-CT/right	6	92.00 ± 4.84	89.83 ± 4.12
IX-CT/left			76.33 ± 4.23
CTX[4]/right	3	90.33 ± 2.96	99.00 ± 2.89
CTX/left			55.67 ± 3.76

[1]Means ± S.E.M., based on the number of rats (n = 3 or 6).
[2]CT, chorda tympani.
[3]IX, glossopharyngeal nerve.
[4]CTX, chorda tympani transected.
[5]n.a., not applicable.

measures ANOVA) showed that there was a large and significant reduction in the number of fungiform papillae on the left side of the CTX group ($F[1,2] = 174.23$, $P = 0.006$) and a small but significant reduction in the CT-CT group ($F[1,2] = 10.89$, $P = 0.021$).

Expression of α-gustducin and the A blood group antigen

The most anterior 5 mm of each tongue was cut into 50-μm sections for immunohistochemical processing. Photomicrographs of fungiform taste buds, shown in Figure 3, demonstrate that the morphology of regenerated taste buds in both the CT-CT and IX-CT groups appeared normal. These sections, which were photographed under Hoffman illumination, show normal fungiform taste buds from the right (unoperated side) of the tongue of a CTX animal (Fig. 3A) and an IX-CT animal (Fig. 3B). Regenerated fungiform taste buds innervated by the CT nerve (Fig. 3C) or the IXth nerve (Fig. 3D) are morphologically indistinguishable from those on the intact side of the tongue. Many of the fungiform papillae on the denervated side of the tongue in CTX animals developed an ectopic filiform-like spine (Fig. 3E), as has been previously described (Oakley et al., 1993). At the anterior tip of the tongue, many taste buds were cut transversely to their long axis, but were still recognizable under Hoffman illumination (Fig. 3F).

Tissue from each animal in the CT-CT, IX-CT, and CTX groups was processed for immunoreactivity to antibodies against α-gustducin and the A blood group antigen. The two sides of the anterior tongue were separated and processed simultaneously, along with tissue from the vallate papilla, which served as a positive immunohistochemical control. The innervation of the right side of the anterior tongue was not disrupted for any of the groups; this tissue provided a comparison to the surgical (left) side. Examples of immunoreactivity for α-gustducin and the A blood group antigen are shown in Figure 4, which shows confocal images of several fungiform and vallate taste buds. Cells immunoreactive (IR) for α-gustducin are shown in red, those for the A blood group antigen in green, and double-labeled cells appear yellow. The taste buds depicted in Figure 4 were chosen to demonstrate immunoreactive cells and are not necessarily representative of the average number of cells per taste bud, which is shown below. For example, a fungiform taste bud from the right (control) side of a cross-regenerated (IX-CT) rat is shown in Figure 4A. There were four α-gustducin-IR cells and no blood group A-IR cells in this taste bud. A antigen expression is seen on the surface of fungiform papillae (Smith et al., 1994b), which appears as a green reaction product at the upper corner of these fungiform images (Fig. 4A–E). Another fungiform taste bud from the right (control) side of a CTX animal is shown in Figure 4B. Here can be seen two α-gustducin-IR cells and one expressing the A antigen, which is distributed on the cell's surface. Fungiform taste buds from the left (surgical) side of a reinnervated (CT-CT) and a cross-reinnervated (IX-CT) animal are shown in Figure 4C and D, respectively, where cells expressing α-gustducin or the A blood group antigen are evident. In Figure 4E is a fungiform taste bud from the right (control) side of an IX-CT animal. In general, these images give the impression that fungiform taste cells are immunoreactive for α-gustducin or the A antigen, but not both, which was confirmed by the cell counts (see below). In Figure 4F, a vallate taste bud from the right side of an IX-CT rat is shown. Many more cells in the vallate taste buds were immunoreactive for α-gustducin and the A blood group antigen and many of these were double-labeled (see below). In this latter animal, the left IXth nerve had been rerouted to the anterior tongue, which would totally denervate only about 20% of the taste buds, predominantly within the left vallate trench (Hosley et al., 1987).

Quantification of labeled cells

The number of cells immunoreactive for α-gustducin and/or the A blood group antigen was determined for every fungiform taste bud in every other 50-μm section of the first 5 mm of the tongue of each animal in the CT-CT, IX-CT, and CTX groups. In addition, all the taste buds in a single section through the vallate papilla of each of three of the CT-CT animals (whose IXth nerve was not disturbed) were counted. The numbers of taste buds and immunoreactive cells in each group are provided in Table 2. Around 40–50 taste buds were analyzed from each side of the anterior tongue of each animal, except for the transected (left) side of the CTX rats, where there were very few remaining taste buds. About 30 taste buds from each of 3 CT-CT animals were counted in the vallate papilla. It is evident from Table 2 that the number of α-gustducin-IR cells in the fungiform taste buds (~ 3) was considerably less than in vallate taste buds (8.37). Similar differences are evident between fungiform and vallate taste buds in the number of blood group A antigen-IR cells and the number of double-labeled cells, both of which were much higher in the vallate taste buds. A one-way ANOVA on the number of α-gustducin cells per taste bud on the unoperated (right) side of the tongue of the CT-CT, IX-CT, and CTX animals demonstrated no significant differences among these groups ($F[2,12] = 0.28$, $P = 0.76$). Therefore, for further statistical analyses, the right sides of each of these groups were combined in order to compare this control side (mean = 3.06 ± 0.10 α-gustducin-IR cells per taste bud, n = 15) against the left (surgical) sides and the vallate papilla.

Figure 5 shows the mean (+S.E.M.) number of immunoreactive cells in the fungiform papillae on the control side of the CT-CT, IX-CT, and CTX groups (filled bars) compared to the left (surgical) sides of these groups (unfilled bars) and the intact vallate papilla of the CT-CT group (hatched bars). If the IXth nerve had any influence over the expression of these antigens in fungiform papillae, then the values for the IX-CT group should be closer to those of the normal target of the IXth nerve, the vallate taste buds. Instead, the values for the IX-CT group were

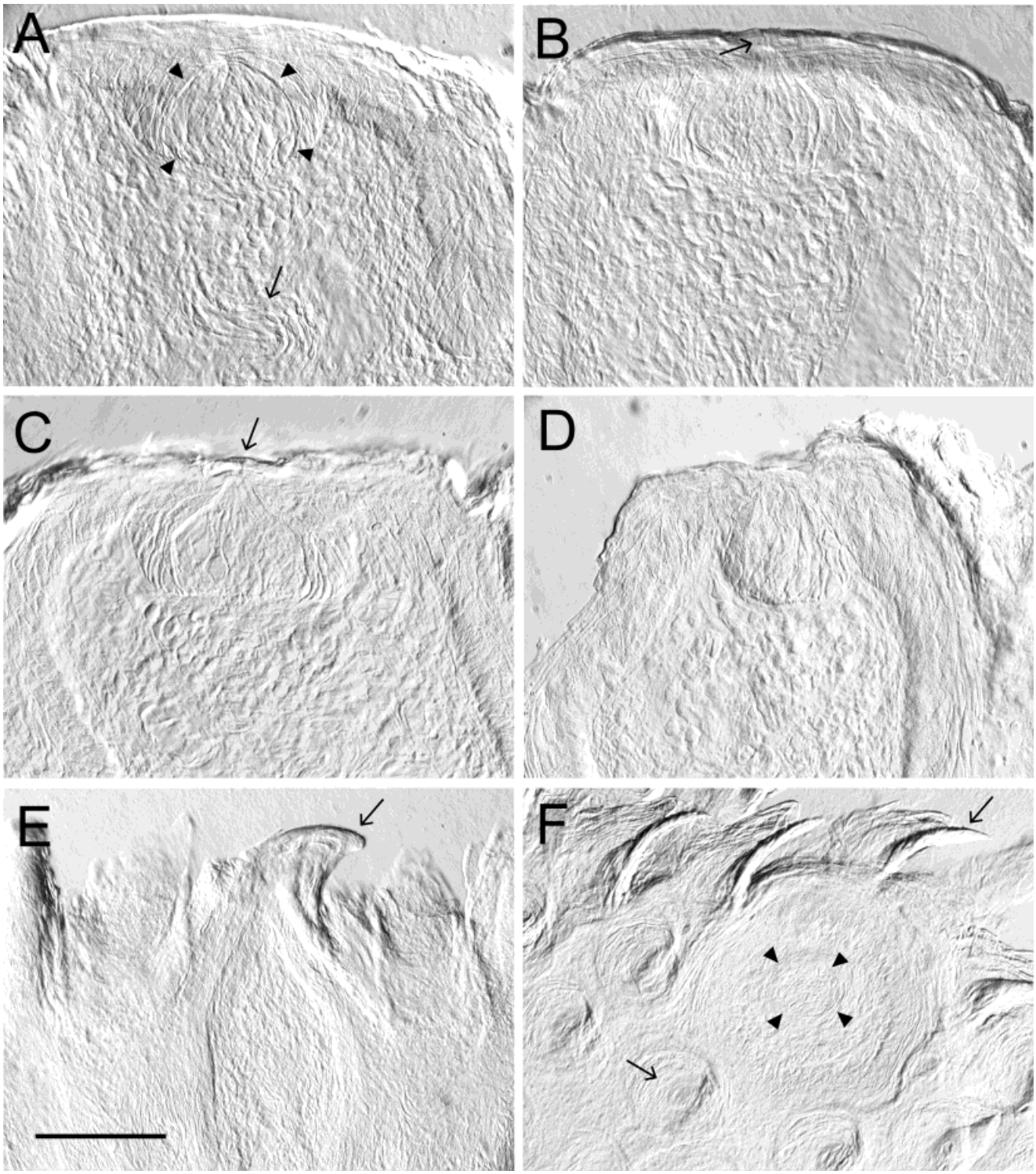

Fig. 3. Photomicrographs of fungiform taste buds under Hoffman modulation contrast optics. **A:** A normal fungiform taste bud from the right (unoperated) side of a chorda tympani transected (CTX) rat; the taste bud is indicated by black triangles and the nerve fibers innervating the taste bud are also visible (arrow). **B:** A normal fungiform taste bud from the right side of a glossopharyngeal nerve (IX)-CT rat; the arrow indicates the position of the taste pore. **C:** Regenerated taste bud on the left side of a chorda tympani (CT)-CT reanastomosed rat; the arrow points to the taste pore. **D:** Regenerated taste bud on the left side of an IX-CT cross-anastomosed rat. **E:** Fungiform papilla from the left (operated) side of a CTX rat, showing an ectopic filiform spine (arrow). **F:** Fungiform taste bud (black triangles) from the right side of a CT-CT reanastomosed rat that was cut transversely to the long axis of the taste bud. Numerous filiform spines are also evident (two are marked by arrows). Scale bar = 50 μm in A–D; 100 μm in E and F.

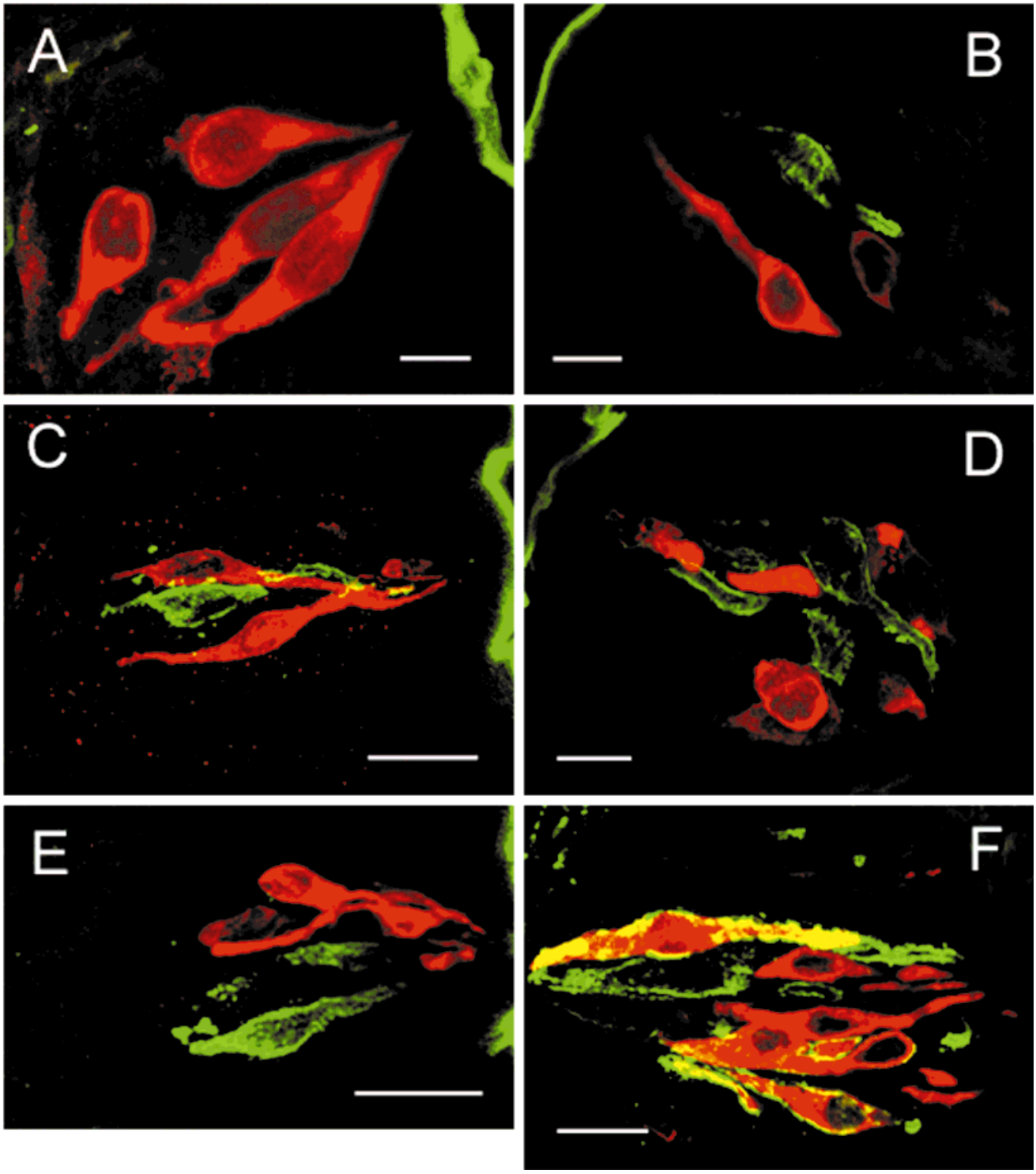

Fig. 4. Confocal images of indirect immunofluoresence in several fungiform taste buds (A–E) and one vallate taste bud (F). Cells immunoreactive for α-gustducin are shown in red, those immunoreactive for the A blood group antigen in green. The green label on the inside edge of each fungiform image is A antigen expression on the surface of the fungiform papilla (see Smith et al., 1994b). **A:** Fungiform taste bud from the right (unoperated) side of a glossopharyngeal nerve (IX)-chorda tympani (CT) cross-anastomosed rat. **B:** Fungiform taste bud from the right (unoperated) side of a chorda tympani transected (CTX) rat. **C:** Fungiform taste bud from the left (operated) side of an CT-CT reanastomosed rat. **D:** Fungiform taste bud from the left (operated) side of an IX-CT cross-anastomosed rat. **E:** Fungiform taste bud from the right (unoperated) side of an IX-CT cross-anastomosed rat. **F:** Vallate taste bud from the right (unoperated) side of an IX-CT rat. Scale bars = 10 μm in A, B, D, and F; 20 μm in C and E.

TABLE 2. Counts[1] of the Number of Immunoreactive Cells/Taste Bud in Each Group

Group/side	Number of rats	Total taste buds	α-gustducin/ taste bud	A blood group/ taste bud	Double-labeled/ taste bud
CT-CT/right	6	272	3.12 ± 0.19	0.25 ± 0.07	0.037 ± 0.009
CT-CT/left		274	3.64 ± 0.23	0.19 ± 0.02	0.028 ± 0.006
IX-CT/right	6	344	2.96 ± 0.18	0.19 ± 0.03	0.045 ± 0.009
IX-CT/left		239	2.53 ± 0.27	0.14 ± 0.03	0.010 ± 0.007
CTX/right	3	149	3.14 ± 0.17	0.26 ± 0.07	0.023 ± 0.003
CTX/left		28	1.31 ± 0.44	0.07 ± 0.04	0
CT-CT/vallate	3	92	8.37 ± 0.80	5.22 ± 0.47	2.71 ± 0.15

[1]Means ± S.E.M., based on the number of rats (n = 3 or 6); left and right indicate fungiform taste buds.
[2]CT, chorda tympani.
[3]IX, glossopharyngeal nerve.
[4]CTX, chorda tympani transected.

not different from those of the control side of the tongue or of the left side of animals whose CT regenerated into the anterior tongue (CT-CT). A one-way ANOVA was conducted on the means for each animal in the groups defined in Figure 5A and there was a significant difference among these groups in the number of α-gustducin-IR cells per taste bud ($F[4,28] = 59.36$, $P < 0.001$). Post-hoc analysis demonstrated that α-gustducin expression differed significantly between the vallate taste buds and all others. Only the vallate taste buds and those in the CTX animals (denervated fungiform papillae) differed significantly from the control counts (Scheffe test, $P < .05$). There was no significant difference among the control side and the reinnervated sides (IX-CT or CT-CT) of the tongue in α-gustducin expression.

Similar analyses were conducted for the data shown in Figure 5B and C. There was a significant difference among these groups in A blood group antigen expression ($F[4,28] = 301.98$, $P < 0.001$). The vallate taste buds had significantly more cells expressing the A antigen than any of the other groups (Scheffe test, $P < .05$); there were no differences among any of the others. A one-way ANOVA on the distribution of double-labeled cells also demonstrated a significant difference among these groups ($F[4,28] = 951.89$, $P < 0.001$), with the counts being significantly higher in the vallate taste buds (Scheffe test, $P < .05$). There was very little blood group A expression in fungiform taste buds and there was absolutely no effect of reinnervation by the IXth nerve on the level of this expression.

DISCUSSION

Fungiform papillae and taste buds are trophically supported by either CT or IXth nerve fibers

Unilateral transection of the CT nerve resulted in a significant loss of taste buds and fungiform papillae on the anterior tongue of the rat (Figs. 1B and 2D, and Table 1). Because the majority of fungiform taste buds are distributed on the anterior tip of the tongue, the greatest number of taste buds were lost from this region. Conversely, those taste buds that remained after CT transection appeared most commonly at the tongue tip, where the mean number of pores was 14.2% of the control side (Fig. 2D). These conclusions are based on using methylene blue dye to demonstrate the presence or absence of taste pores on the fungiform papillae, a method that has been used previously to quantify taste buds following CT transection in the rat (St. John et al., 1995). Denervation of the fungiform papillae results in structural changes to the taste pore (Parks and Whitehead, 1998). The loss of taste buds on the operated side of the CTX rats was also confirmed in the present study in the histological sections, where the number of taste buds on the cut side was 18.8% of that found on the intact side (Table 2). The proportion of fungiform taste buds found in the rat after 100 days following CT lesion (23.4%; Hård af Segerstad et al., 1989) is near the value determined by either method (methylene blue or histology) in our CTX animals, which survived 90.33 ± 2.96 (S.E.M.) days after surgery.

We made no attempt to determine the status of innervation of these residual taste buds in the CTX animals. Previous experiments have suggested that residual fungiform taste buds in gerbil and rat may derive some innervation from other sources, especially following the long survival times used in the present experiment (Kinnman and Aldskogius, 1988; Hård af Segerstad et al., 1989; Oakley et al., 1993), although the source of such innervation is unknown. The procedure of avulsing the CT nerve from the middle ear makes it unlikely that these taste buds were reinnervated by the ipsilateral CT. This conclusion is supported by the significant reduction in the number of α-gustducin-expressing cells on the operated side of CTX animals compared to those on either the unoperated side of the tongue or on the regenerated side of the CT-CT rats (Fig. 5A, Table 2). That is, if the CT reinnervated these taste buds, they should have expressed the normal number of α-gustducin-positive cells, as in the CT-CT animals.

In both the CT-CT and the IX-CT animals, the number of fungiform papillae following reinnervation was reduced on the operated side of the tongue. Regeneration of the CT nerve resulted in an average of about 83% regeneration of taste pores and the reinnervation of the anterior tongue by the IXth nerve produced about 75% of the normal complement of taste pores. This amount of regeneration is somewhat better than that seen after cutting the rat CT nerve within the middle ear without subsequent surgical reattachment, which resulted in the number of taste buds reaching a maximum of about two-thirds of normal by 50 days after transection (St. John et al., 1995). These same investigators also reported a loss of fungiform papillae on the transected side of the tongue (St. John et al., 1995). Thus, regeneration of fungiform taste buds following reinnervation, by either the CT or the IXth nerve, is less than complete. Nevertheless, innervation by either nerve serves to maintain both fungiform papillae and their taste buds, albeit at somewhat lower numbers.

Although we have no independent verification that taste buds in the IX-CT animals were innervated by fibers of the IXth nerve, there are several results supporting that conclusion. First, the CTX animals had significantly fewer fungiform taste buds than any of the other surgical groups or the unoperated side of the tongue (Fig. 2D, Table 2), suggesting that avulsing the CT prevented regeneration. Second, in the CTX rats the number of cells expressing α-gustducin in the remaining taste buds was significantly reduced (Fig. 5A, Table 2), indicating that they were not successfully reinnervated by the CT. These results make it highly unlikely that taste buds in the IX-CT animals were somehow reinnervated by the CT. Finally, in each of the IX-CT rats, the physical integrity of the IX-CT anastomosis was confirmed prior to perfusion, indicating that any fibers regenerating through the distal CT would have

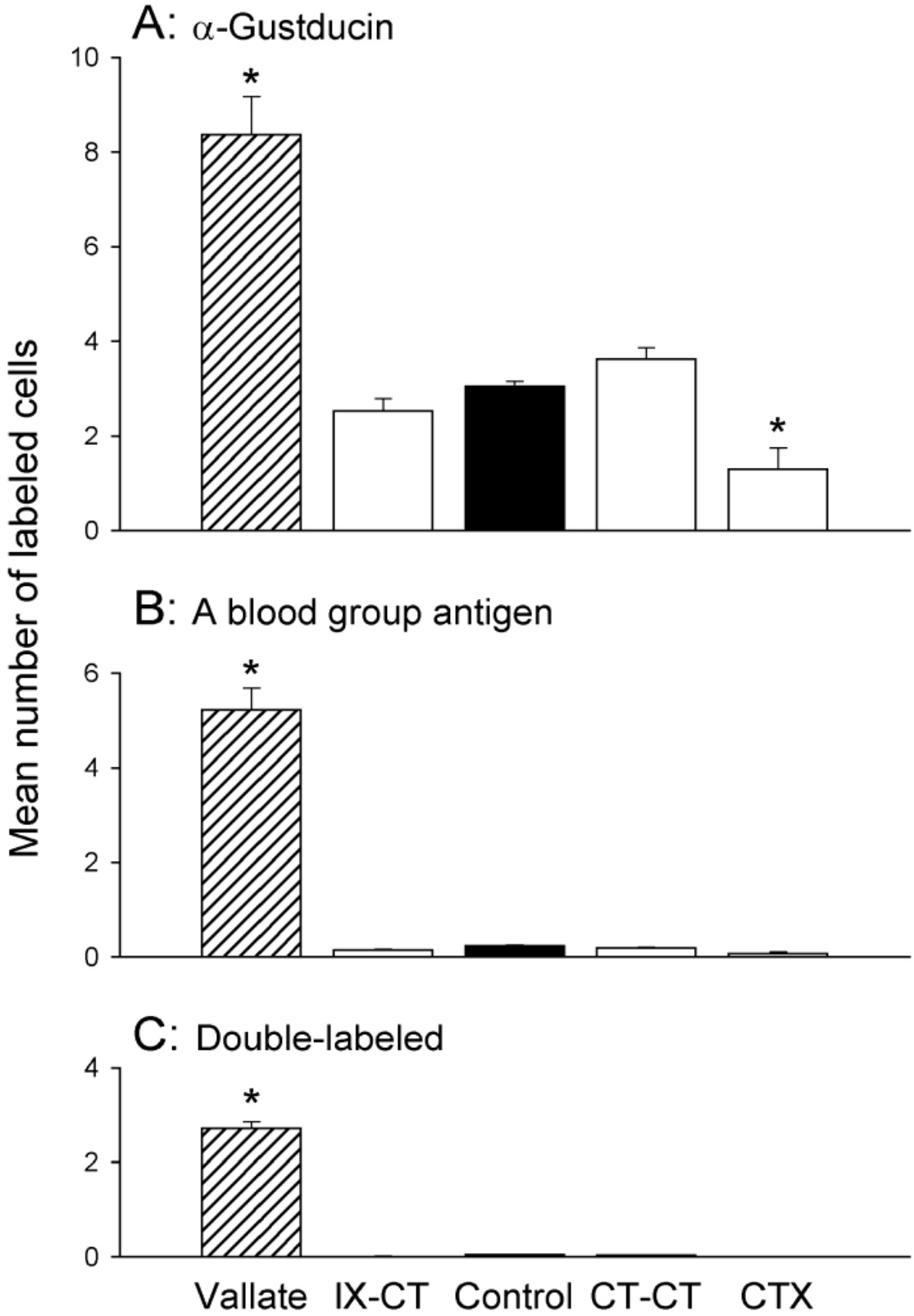

Fig. 5. Mean (+S.E.M.) number of labeled cells in taste buds of the vallate papillae of chorda tympani (CT)-CT rats (hatched bars) and in fungiform papillae of the left (operated; open bars) and right (control; solid bar) sides of the glossopharyngeal nerve (IX)-CT, CT-CT, and chorda tympani transected (CTX) rats. **A:** Numbers of α-gustducin-immunoreactive cells. **B:** Numbers of cells expressing the A blood group antigen. **C:** Numbers of cells that were double-labeled for both α-gustducin and the A antigen. Asterisks indicate that the vallate counts were significantly higher than all others and that the CTX counts were significantly less than control (see text).

arrived there via the proximal IXth nerve. These considerations make it implausible that the regenerated fungiform taste buds in the IX-CT animals were innervated by any source other than the IXth nerve.

Molecular markers characterize differentiated taste cells

Previous studies have shown that vallate taste buds and the molecular markers specific to them are trophically dependent upon their innervation. Transection of the glossopharyngeal nerve results in degeneration of the vallate taste buds (Guth, 1957), which reappear after the nerve regenerates into the tongue (Iwayama and Nada, 1969; Hosley et al., 1987). The taste cell expression of the neural cell adhesion molecule (NCAM), several blood group antigens, and other taste-cell specific molecular markers disappears after bilateral crush of the IXth nerve (Smith et al., 1993, 1994b). Regeneration of the IXth nerve results in differentiation of the gustatory epithelium into taste buds and the expression of NCAM by a subset of the taste cells (Smith et al., 1994a). The vallate taste epithelium does not express these taste cell-specific molecular markers in the absence of morphologically identifiable taste buds, which themselves depend upon innervation for their differentiation and maintenance.

An exception to these observations has been noted for fungiform taste buds, a few of which remain in an atrophic state following denervation in rats (Hård af Segerstad et al., 1989; Oakley et al., 1993), hamsters (Whitehead et al., 1987; Oakley et al., 1993), and gerbils (Oakley et al., 1993). Although there is considerable debate about whether these remaining taste buds are nevertheless innervated by some unknown source, their morphology is different from normally innervated taste buds. Atrophic taste buds are 50% smaller in volume than normally innervated taste buds and lack a visible pore (Oakley et al., 1993). Although most authors refer to these remaining taste buds as atrophic or "remnant" taste buds, others make a distinction, reserving the term "remnant" for collections of cells that were merely a fragment of a fungiform taste bud, without the characteristic oval shape seen in atrophic buds (Oakley et al., 1993). Nevertheless, such remaining taste buds are seen only in fungiform papillae and have never been described in vallate or foliate papillae, where denervation results in a complete loss of recognizable taste buds in any form.

Although atrophic fungiform taste buds are smaller than normal, they nevertheless contain a few cells which express molecules characteristic of differentiated taste cells, including keratin 19 (Oakley et al., 1993), NCAM and neuron-specific enolase (Whitehead et al., 1998). The numbers of cells expressing these markers is reduced in denervated taste buds. Because the results of the present experiment show that the number of cells expressing α-gustducin in reinnervated taste buds is the same as in normal ones (Table 2 and Fig. 5A), the significant decrease in the number of cells expressing α-gustducin in fungiform taste buds of the CTX animals suggests that these taste buds are not innervated normally and are most likely atrophic buds rather than regenerated ones.

Taste cell phenotype is determined by the epithelium

Reinnervation of the anterior tongue by fibers of the IXth nerve resulted in regeneration of fungiform taste buds. These regenerated buds were similar in appearance to those on the intact side of the tongue or to those that regenerated after reinnervation by the CT nerve (Fig. 3). Within the normal rat fungiform papillae, taste buds contained approximately three cells expressing the gustatory G protein, α-gustducin (Fig. 5A, solid bar). The number of α-gustducin-expressing cells was significantly greater in the vallate taste buds (Fig. 5A, hatched bar), as shown previously for the rat (Boughter et al., 1997). Almost three times as many cells in each vallate taste bud expressed this marker in comparison to the fungiform taste buds. When fungiform taste buds regenerated following reinnervation by either the CT nerve or the IXth nerve (Fig. 5A, open bars), there was no significant difference between the number of α-gustducin-positive cells compared to the contralateral (control) side of the tongue (Fig. 5A, solid bar). If the molecular phenotype of fungiform taste cells were determined by the innervating nerve, then the number of cells expressing α-gustducin in the IX-CT rats would be expected to increase to the level seen in the vallate taste buds, which are normally innervated by the IXth nerve. Instead, the number of α-gustducin-expressing cells in fungiform taste buds reinnervated by the IXth nerve is exactly the same as that in intact fungiform taste buds or those reinnervated by the CT nerve. These data suggest strongly that the molecular expression of this G protein subunit is determined by the epithelium from which the cells arise, independent of their source of innervation.

The A blood group antigen is a cell-surface carbohydrate epitope that is expressed by many fewer fungiform taste cells than vallate (Fig. 5B). This and other human blood group epitopes are expressed by subsets of cells in fungiform, vallate, and foliate taste buds (Smith et al., 1994b). Cell-surface carbohydrates have been implicated in cell-cell recognition and cellular interactions in the developing nervous system (Dodd and Jessell, 1985; Mollicone et al., 1985; Gil-Loyzaga et al., 1989; Jessel et al., 1990; Zipser, 1995). Thus, we might expect that the A blood group antigen would have a very different role in taste bud cell function than α-gustducin, which is a G protein subunit thought to be involved in the transduction of sweet and bitter taste stimuli (McLaughlin et al., 1992; Wong et al., 1996). The A blood group antigen or other carbohydrates could play roles in cell-cell recognition important during cell turnover and synaptogenesis or in the morphological integrity of the taste bud. In such a role, these markers might be expected to be influenced by the innervating nerve as the taste buds are reformed following nerve fiber regeneration. Nevertheless, denervation of the fungiform taste buds and subsequent reinnervation by the IXth nerve, which normally innervates taste buds of the vallate papillae, which contain significantly more A-expressing cells, had no effect on the number of fungiform taste bud cells expressing this antigen.

These immunocytochemical results extend those of previous electrophysiological studies that have shown that the sensitivity of taste fibers in cross-regenerated nerves is determined by the epithelium (Oakley, 1967; Nejad and Beidler, 1987). These data demonstrate that the phenotype of taste bud cells is determined by the epithelium from which the cells arise and is not controlled by factors associated with the innervating nerve fibers. Although the differentiation and maintenance of taste bud cells is trophically dependent on an intact innervation, with the exception of a few remnant fungiform taste cells, the

particular molecular phenotype of the differentiated taste cells is independent of the source of this innervation. Thus, the neural factors important for triggering taste cell differentiation are distinct from factors that determine gene expression in these cells.

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