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Neuron/Target Plasticity in the Peripheral Gustatory System

MARSHALL G. SHULER,[1] ROBIN F. KRIMM,[2] AND DAVID L. HILL[3]*
[1]Picower Center for Learning & Memory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
[2]Department of Anatomical Sciences and Neurobiology, University of Louisville School of Medicine, Louisville, Kentucky 40292
[3]Department of Psychology, University of Virginia, Charlottesville, Virginia 22904

ABSTRACT

Taste bud volume on the anterior tongue in adult rats is matched by an appropriate number of innervating geniculate ganglion cells. The larger the taste bud, the more geniculate ganglion cells that innervate it. To determine if such a match is perturbed in the regenerated gustatory system under different dietary conditions, taste bud volumes and numbers of innervating neurons were quantified in adult rats after unilateral axotomy of the chorda tympani nerve and/or maintenance on a sodium-restricted diet. The relationship between taste bud size and innervation was eliminated in rats merely fed a sodium-restricted diet; individual taste bud volumes were smaller than predicted by the corresponding number of innervating neurons. Surprisingly, the relationship was disrupted in a similar way on the intact side of the tongue in unilaterally sectioned rats, with no diet-related differences. The mismatch in these groups was due to a decrease in average taste bud volumes and not to a change in numbers of innervating ganglion cells. In contrast, individual taste bud volumes were larger than predicted by the corresponding number of innervating neurons on the regenerated side of the tongue; again, with no diet-related differences. However, the primary variable responsible for disrupting the function on the regenerated side was an approximate 20% decrease in geniculate ganglion cells available to innervate taste buds. Therefore, the neuron/target match in the peripheral gustatory system is susceptible to surgical and/or dietary manipulations that act through multiple mechanisms. This system is ideally suited to model sensory plasticity in adults. J. Comp. Neurol. 472:183–192, 2004.

Indexing terms: taste; geniculate ganglion; taste buds; retrograde labeling; chorda tympani nerve; nerve section

In vertebrates, the maintenance of specialized receptor cells located in sensory epithelia depends on the presence of sensory nerves (Jacobsen, 1971; Zelena, 1976). For mammalian taste buds, organized cellular structure rapidly disappears following denervation and reappears upon reinnervation (e.g., Cheal and Oakley, 1977). When gustatory nerves are sectioned, taste buds degenerate and subsequently regenerate after innervation is restored (Guth, 1957; Fujimoto and Murray, 1970; Cheal and Oakley, 1977). Although there is some debate whether denervated taste buds disappear or merely take on a "quiescent" appearance (Whitehead et al., 1987), it is clear that there is a significant morphological change from their predenervated configuration (Oakley et al., 1993). Even after the nerve supply is restored, long-term changes in taste bud morphology are evident (St. John et al., 1995). By contrast, a more severe change in peripheral gustatory morphology occurs when axotomies are performed early in development (Sollars and Bernstein, 2000; Sollars et al., 2002). Early postnatal chorda tympani nerve section leads to a lack of regeneration of the nerve and of the target

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*Correspondence to: David L. Hill, Department of Psychology, PO Box 400400, University of Virginia, Charlottesville, VA 22904. E-mail: dh2t@virginia.edu

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taste bud, resulting in a permanent loss of fungiform taste buds and papillae (Sollars et al., 2002). The developmental findings underscore the trophic dependence between these two structural components.

The period during which taste receptor cells become functional following nerve regeneration may be a time when they are especially susceptible to environmental influences (i.e., they are "plastic"). Indeed, dietary manipulations influence the responses of newly functioning taste receptors following neural regeneration. Chorda tympani nerve taste responses to sodium salts from the regenerated nerve in rats fed a sodium-restricted diet as adults are suppressed selectively compared to controls. Surprisingly, alterations in taste-elicited responses from the chorda tympani nerve on the contralateral, intact side of the tongue are also present (Hill and Phillips, 1994). Responses to sodium salts are highly attenuated soon after sectioning of the contralateral nerve in sodium-restricted rats and then increase gradually during the next 50 days, after which responses are supersensitive compared to controls. Furthermore, the early effects seen in the intact nerve in rats with combined axotomy and dietary sodium restriction may relate to altered immune function (Phillips and Hill, 1996). In fact, responses from the chorda tympani nerve to sodium salts can be altered even by localized tissue damage to the contralateral side of the tongue (Hendricks et al., 2004).

The widespread functional changes induced by these experimental manipulations underscore how this is an ideal system to study sensory plasticity. Indeed, the anatomical features of the mature and intact gustatory system have been used in the past to promote it as a model system for such experiments (e.g., Beidler and Smallman, 1965). Namely, taste bud cells turnover and are replaced on a cycle of about 10 days, requiring synapse replacement or reorganization to maintain reliable afferent messages over time. Recent anatomical data have further provided a way to explore the organization of nerve/target relationships for individual taste buds. Specifically, there is a highly predictable relationship between the volume of a taste bud and the number of neurons that innervate the taste bud (Krimm and Hill, 1998). Simply stated, the larger the taste bud, the more neurons that innervate it. Not only does this match demonstrate the orderliness in the mature peripheral taste system in the face of taste bud cell turnover, but it can also be used as a standard function to compare with data from experimental groups. As examples, the relationship develops normally over the first 40 postnatal days (Krimm and Hill, 1998) and can be disrupted by feeding rats a low-sodium diet during pre- and postnatal development (Krimm and Hill, 1999). Therefore, there are clear developmentally related components of this match. The results of the current study add to these findings by examining these neuron/target interactions in a system that is disrupted at adulthood, leading to wholesale degeneration and subsequent regeneration of target taste buds and to the potential degeneration of innervating neurons. Our findings complement the existing functional data and collectively highlight the adult peripheral gustatory system as a model for studies of sensory plasticity.

MATERIALS AND METHODS

The number of geniculate ganglion cells that innervate single taste buds was determined in rats that had either received unilateral chorda tympani section and/or dietary sodium restriction. The number of neurons innervating single taste buds was found by labeling individual fungiform papillae with fluorescent retrograde neuronal tracers that were transported from the labeled taste bud to cells of the chorda tympani nerve in the geniculate ganglion. Since each fungiform papilla in rats usually contains only one taste bud, the total number of labeled geniculate ganglion neurons is assumed to be the number of neurons that innervate a single taste bud. Accompanying data concerning the number of neurons surviving unilateral chorda tympani nerve section were provided through bulk labeling the chorda tympani nerve with a fluorescent tracer and then counting the number of labeled cells in the geniculate ganglion. Table 1 describes the overall experimental design with the respective numbers of observations. All experiments were endorsed by the Animal Care and Use Committee of the University of Virginia and followed guidelines set by the National Institutes of Health.

TABLE 1. Experimental Design Showing the Experimental Group, Experiment, Side of Tongue Analyzed (Left-Uncut; Right-Regenerated), and Number of Observations (n) for Each Group

	No. of observations					
	Single papilla label		Taste bud measurements		Geniculate ganglion counts	
Group	Left	Right	Left	Right	Left	Right
Cut + diet	18	23	45	48	6	6
Cut only	14	18	28	62	6	6
Diet only	23		80		5	
Controls	22		39		7	

Unilateral chorda tympani nerve section and dietary manipulation

Male and female Sprague-Dawley rats purchased from Harlan Sprague Dawley (Indianapolis, IN) were 40–60 days old at the time of nerve section and/or dietary manipulation. The surgical procedure was the same as described by Hill and Phillips (1994). Briefly, rats receiving nerve section were initially injected with 0.1 ml atropine sulfate (0.54 mg/ml, i.p.) and subsequently anesthetized with sodium Brevital (60 mg/kg, i.p.). The right chorda tympani nerve was exposed in the neck and sectioned between the anterior belly of the digastric and the masseter muscles where the chorda tympani nerve bifurcates from the lingual branch of the trigeminal nerve. Thus, the lingual branch of the trigeminal nerve remained intact. Wounds were closed with sutures and the animals were allowed to recover on a water-circulating heating pad.

Rats receiving nerve sections were then either injected with furosemide (two injections of 10 mg each within 24 hours) and placed on a sodium-deficient diet (0.03% NaCl; ICN Biochemicals, Costa Mesa, CA) throughout the experiment or were continued on standard laboratory chow (1.0% NaCl) and distilled water. An additional two groups of rats were either injected with furosemide and placed on the sodium-deficient diet but received no nerve section or received no dietary manipulation and no nerve section. Therefore, this study reports findings from four groups: 1) unilateral chorda tympani nerve section followed by a low sodium diet (cut + diet); 2) unilateral chorda tympani

nerve section with a sodium replete diet (cut only); 3) intact chorda tympani nerve with a low sodium diet (diet only); and 4) intact chorda tympani nerve with a sodium replete diet (controls). The control and diet-only groups received a sham surgery in that they underwent all of the surgical procedures except unilateral nerve section.

Fluorescent labeling of single fungiform taste buds

The procedure for labeling neurons innervating single fungiform taste buds is described in detail in Krimm and Hill (1998) and was used here. In all groups (see Table 1), rats were anesthetized with sodium pentobarbital (50 mg/kg; 1 cc/kg) at 100–110 days (50–60 days after nerve section and/or onset of dietary restriction). The period elapsing from nerve section to papilla labeling was chosen to ensure that the sectioned nerves regenerated to support taste bud structures and functional taste responses (Hill and Phillips, 1994). Following anesthetization, the dorsal, anterior half of the tongue was exposed from the mouth by gently pulling on the ventral tongue. The tongue was then stabilized by pressing the ventral surface to a glass slide covered with putty. Visualization of fungiform papillae and taste pores were enhanced by painting the tongue surface with a 0.5% aqueous solution of methylene blue (Fisher Scientific, Pittsburgh, PA). We did not notice an appreciable absence of taste pores in any of the preparations, including rats in which the chorda tympani nerve regenerated. Using a micromanipulator and surgical microscope, a glass pipette (~150 μm diameter) containing one of the two fluorescent retrograde tracers was placed over the target papilla, creating an electrical seal without penetrating the epithelium. A small wire (0.3 mm dia.) was inserted into the ventral tongue as the reference electrode. By applying a square, anodal pulse, one of two fluorescent tracers was iontophoresed into the target papilla. The two fluorescent tracers were Fluoro-Gold (2% mixed in distilled water; Fluorochrome, Englewood, NJ; 0.7 μA positive current; 4 seconds on / 4 seconds off for 4–5 minutes) and tetramethylrhodamine dextran (3,000 MW, 10% mixed in distilled water; Molecular Probes, Eugene, OR; 4.0 μA positive current; 4 seconds on / 4 seconds off for 8 minutes). To maximize the amount of information obtained from each rat and to be able to assess innervation patterns of taste buds on both sectioned and intact sides of the tongue, a papilla on each side of the tongue was labeled. Only papillae on the tongue mid-region were labeled so that comparisons with earlier work (Krimm and Hill, 1998) could be readily accomplished. The mid-region of the tongue was defined as located between 2.5 and 7.5 mm rostral to the intermolar eminence (Krimm and Hill, 1998). It should be noted that the size of the taste bud could not be determined at the time of fluorescent tracer injection; rather, taste bud volume was assessed only following histological processing (see next section). Upon completion of the label, a map of the dorsal tongue was made by using measurements and lingual landmarks so that the labeled papillae could later be reliably identified.

Histological procedures

Rats were sacrificed with a lethal dose of urethane (0.9 mg/kg) 3 days after labeling, and were perfused intracardially with a Krebs solution, followed by 4% paraformaldehyde in an acetate buffer (pH 6.5), followed by 4% paraformaldehyde in a borate buffer (pH 9.5). The tongue and geniculate ganglia were removed and placed in 30% sucrose overnight. Serial, 8-μm sections of the geniculate ganglia and 20-μm sections of the tongue containing labeled papillae were cut with a cryostat, thaw-mounted on gelatin-coated glass slides, cleared with xylenes, and coverslipped with DPX (Aldrich, Milwaukee, WI).

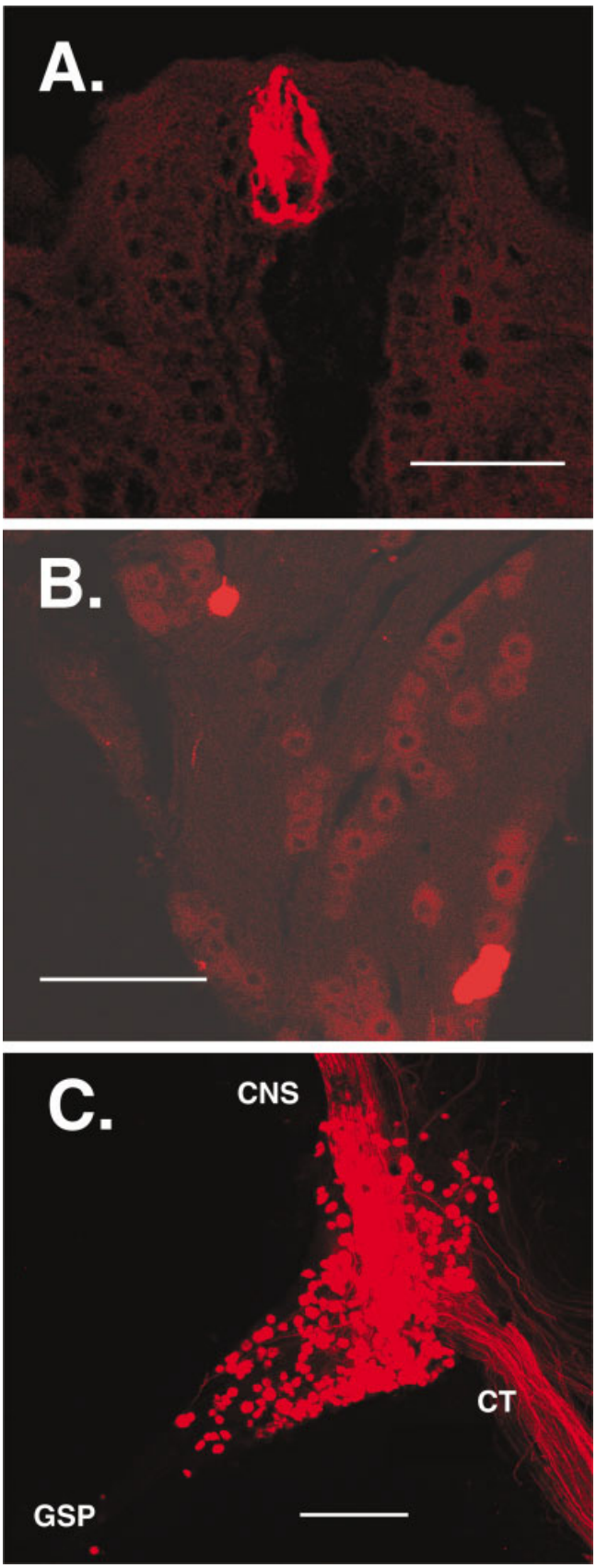
Tissue was examined with an epifluorescent microscope (Olympus BH-2). Fluoro-Gold labels were visualized by using a wideband ultraviolet excitation and tetramethylrhodamine labels were examined using a wideband green light excitation. The labels from both tracers in the tongue and geniculate ganglion were robust and were easily distinguished from background fluorescence (Fig. 1A,B). Therefore, an "all-or-none" criterion was used to determine if a cell was fluorescently labeled. Tongues were examined initially to determine that taste bud-containing papillae were sufficiently, but not excessively, labeled. Only ganglia in which the fluorescent label was restricted to the dorsal half of the fungiform papilla were used in data analyses (Krimm and Hill, 1998); this ensures that neurons not innervating the papillae but with fibers located in the neural plexus at the base of papillae were not labeled. The number of ganglion cells labeled with Fluoro-Gold or with tetramethylrhodamine were simply counted in ganglia meeting this criterion. After determining the extent of fluorescent label within the taste bud, coverslips were removed and slides containing tongue sections were stained with hematoxylin and eosin (H&E).

Volume measurements of fungiform taste buds

In order to fully examine taste bud volumes on the sectioned and uncut sides of the tongue for each group, an additional three rats for each group were prepared as noted above. However, labeling of single papillae was not carried out. This allowed us to readily sample from a larger group of taste buds compared to those labeled with a fluorescent tracer. Rats were overdosed with a lethal dose of urethane (0.9 mg/kg), perfused, and then tongues were sectioned and then stained with H&E for analysis of taste bud volumes. Volume measurements were made with an Olympus Cue-2 image analysis system. As done for the single papilla labeling experiments and consistent with our previous work (Krimm and Hill, 1998; 1999), only taste buds in the mid-region of the tongue (between 2.5 and 7.5 mm rostral to the intermolar eminence) were analyzed. To be consistent with other reports, the borders were drawn such that peripheral cells of the taste bud were included in the measurement (Whitehead et al., 1985; Krimm and Hill, 1998). Taste bud areas were multiplied by the section thickness (20 μm) and summed to calculate the taste bud volume (see Table 1; Krimm and Hill, 1998; 1999).

Labeling and counts of chorda tympani nerve cells in the geniculate ganglion

To determine if there was an axotomy-related loss in chorda tympani neurons, 12 additional rats sustained unilateral chorda tympani nerve section and were maintained on a sodium-replete diet (cut only, n = 6) or were sodium-restricted as described above (cut + diet, n = 6; see Table 1). Approximately 50 days following unilateral nerve section, rats were anesthetized with ketamine/xylazine (150 mg/kg body wt; IM) and both the regenerated (right) and uncut (left) chorda tympani nerves were



cut and bulk labeled with tetramethylrhodamine dextran crystals (Molecular Probes, Eugene, OR). Age-matched controls (n = 7) and diet-only rats (n = 5) had their right chorda tympani labeled similar to the other two groups. Access to the chorda tympani nerves was gained through dissection and retraction of the anterior and posterior digastric muscles from the ventral surface of the tympanic bulla. A small hole was made in the tympanic bulla, exposing the chorda tympani nerve proximal to the auditory ossicles. The chorda tympani nerves were sectioned and labeled with the fluorescent tracer. Wounds were closed with sutures and the animals were allowed to recover on a water-circulating heating pad. Following a 24-hour survival, rats were sacrificed with an overdose of urethane (0.9 mg/kg) and perfused with PBS (pH 7.2) followed by 4% paraformaldehyde. Both left and right geniculate ganglia were removed in cut-only and cut + diet rats, the right ganglia were removed from controls and diet only rats; all ganglia were postfixed overnight. It should be noted that the site of bulk labeling was ~1 cm from the site of the original nerve section. Therefore, scar tissue resulting from the first section did not influence bulk labeling of the nerve.

Each geniculate ganglion was trimmed from the adjacent facial nerve with the aid of a fluorescent dissecting microscope (Leica MZFLIII), ensuring that the entire ganglia with all of the labeled cells remained intact. Whole ganglia (~120 μm in depth) were placed on a glass coverslip, covered with PBS/glycerol, and then covered with another coverslip. The entire ganglion was then imaged at optical sections of 2 μm with an Olympus Fluoview 3 laser confocal microscope (Fig. 1C). Image stacks were stored and subsequently imported into Neurolucida software system (MicroBrightField, Colchester, VT) for counting each labeled neuron. Briefly, optical images were viewed and labeled neurons were marked through an optical overlay. By comparing successive images we were able to count each labeled cell. The side of the ganglia (regenerated vs. uncut) and dietary manipulation done to the rat (sodium-replete vs. sodium-restricted) were blind to the experimenter during collection of the data. PhotoShop (Adobe Systems, San Jose, CA) was used to compose digital images. Only contrast and brightness were used to enhance the images.

Data analysis

Volumes of labeled taste buds visualized with H&E (i.e., not fluorescently labeled) for animals within each group (n = 3/group) were analyzed with analysis of variance (ANOVA) to determine if there were significant interani-

Fig. 1. **A:** Photomicrograph of a coronal section through taste bud labeled with tetramethylrhodamine dextran amine 3 days before sacrificing the rat. The fungiform papilla can be seen by the less intensely imaged red cells and the taste bud is shown in bright red. **B:** Photomicrograph of two tetramethylrhodamine dextran amine-labeled geniculate ganglion cells labeled by the injection shown in A. **C:** Photomicrograph of a geniculate ganglion containing cells labeled with tetramethylrhodamine dextran amine by bulk labeling the entire chorda tympani nerve. The entire ganglion was imaged with a laser confocal microscope; optical sections were collapsed to make this image. All tissue are from control animals. CT, chorda tympani nerve; GSP, greater superficial nerve; CNS, central stump. Scale bars = 25 μm in A; 100 μm in B; 200 μm in C.

mal variability. None of the comparisons were significant ($P > 0.10$); therefore, taste bud volumes were pooled across animals within each group (see Table 1). The pooled taste bud volumes were compared to the volumes of fluorescently labeled taste buds measured from the same region by using *t*-tests to determine if the sample of taste buds labeled with fluorescent tracers represented the larger population of unlabeled taste buds. ANOVA was used to compare mean taste bud size, mean number of ganglion cells labeled with single-papilla injections, and mean numbers of bulk-labeled ganglion cells among experimental groups. Following a significant ANOVA, multiple comparisons were made by using the Fisher's PLSD posttest. Only the probability of each posttest is presented in the results. Pearson product-moment correlations were used to analyze the relationship between taste bud size and number of innervating ganglion cells. The alpha level was set at $P < 0.05$ for statistical comparisons.

RESULTS

Labeled taste buds are representative of the population

Mean volumes of taste buds labeled with the fluorescent labels were not significantly different than the respective mean volumes calculated from the larger population of H&E-stained taste buds. The only comparison that approached significance was for the diet-only group, in which the labeled taste bud mean was 7.5 $\mu m^3 \times 10^4$ and the mean from the larger sample of taste buds was 6.3 $\mu m^3 \times 10^4$ ($P = 0.06$); all other comparisons did not approach significance ($P > 0.10$).

It should be noted that some statistical characteristics of the two distributions, other than the means, appeared different. This is most noticeable for controls in which the range for fluorescently labeled taste buds were shifted toward the smaller volumes. We attribute this in part to the random selection of taste buds fluorescently labeled in the mid-region of the tongue (see Materials and Methods) and the larger number of rats represented in the fluorescently labeled group. Interestingly, taste bud volumes from H&E-stained tissue measured by us previously (Krimm and Hill, 1998) match both the range of the current fluorescently labeled tissue and the mean of the current H&E-stained tissue (7.9 $\mu m^3 \times 10^4$ vs. 8.0 $\mu m^3 \times 10^4$; Fig. 2, and see below). More taste buds were measured in the earlier study (50 vs. 39); therefore, increasing the number in the current sample may have also revealed smaller taste buds here. Since control data from H&E-stained taste bud volumes match our earlier observations (Krimm and Hill, 1998), they represent a larger sample than fluorescently labeled taste buds in all groups, and the sampling procedures are consistent among the groups (e.g., n = 3 rats/group), we chose to use data only from the H&E-stained tissue to make statistical comparisons among groups for taste bud volume.

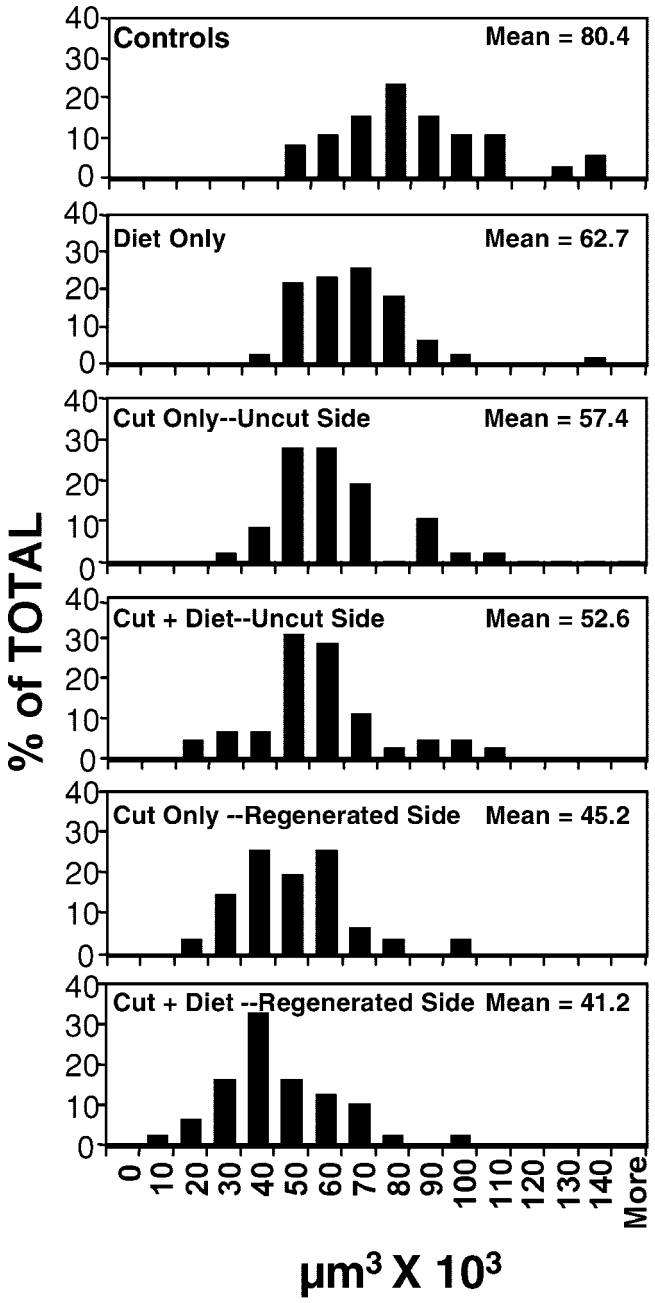


Fig. 2. Histograms showing the proportion of total taste buds of different volumes for controls, diet control rats, the intact side of the tongue in cut-only rats (Cut Only – Uncut Side), the uncut side of the tongue in cut + diet rats (Cut + Diet – Uncut Side), the regenerated side of the tongue in cut-only rats (Cut Only – Regenerated Side), and the regenerated side of the tongue in cut + diet rats (Cut + Diet – Regenerated Side).

Taste bud volumes

There was a progressive decrease in taste bud volumes related to dietary manipulation and to the surgical manipulation (Fig. 2). Merely placing rats on a sodium-restricted diet or unilateral sectioning the chorda tympani nerve reduced taste bud volumes, even on the unoperated side of the tongue. Mean (±SEM) taste bud volume in controls was 8.0 ± 0.4 $\mu m^3 \times 10^4$. The mean taste bud volume for diet-only rats was 6.3 ± 0.2 $\mu m^3 \times 10^4$ and was significantly less than that in controls ($P < 0.0001$). By comparison, the mean taste bud size on the uncut side of the tongue in cut-only rats was 5.7 ± 0.3 $\mu m^3 \times 10^4$, also significantly less than for controls ($P < 0.000001$; Fig. 2). Combining sodium restriction with a unilateral nerve sec-

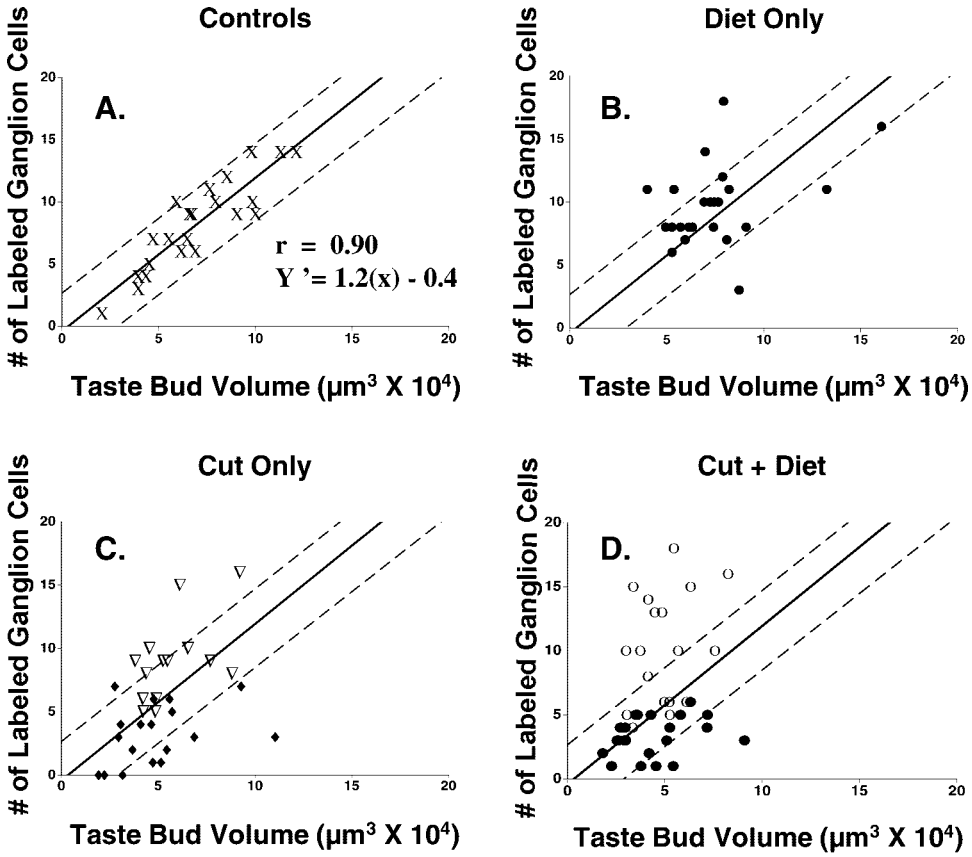


Fig. 3. Number of labeled geniculate ganglion cells plotted against taste bud volumes for control rats (**A**), diet-only rats (**B**), cut-only rats (**C**), and cut + diet rats (**D**). The solid line represents the linear regression from controls and 95% confidence intervals are displayed with dashed lines. The correlation coefficient and the linear regression equation for controls are shown in the bottom right of A. Open symbols are data from the uncut side of the tongue and solid symbols are data from the regenerated side of the tongue in C and D.

tion also resulted in a significant decrease in taste bud size on the uncut side compared to controls (Fig. 2: Cut + Diet – Uncut Side; Mean = 5.3 $\mu m^3 \times 10^4 \pm 0.3$; $P < 0.000001$); however, the mean was similar to the uncut side in cut-only rats (Cut Only – Uncut Side; Mean = 5.7 $\mu m^3 \times 10^4 \pm 0.3$; $P > 0.10$). Therefore, there is not an additive effect on the uncut side of section and diet. Together, these findings demonstrate that sectioning the chorda tympani nerve reduces taste bud size on the opposite, uncut, side of the tongue.

The largest effects on taste bud volume occurred on the sectioned (i.e., regenerated) side of the tongue (Fig. 2). Taste buds on the regenerated side of the tongue in cut-only rats were 59% of the control mean and were 51% of the control mean in cut + diet rats (4.5 ± 0.2 and 4.1 ± 0.2 $\mu m^3 \times 10^4$, respectively; $P < 0.0001$). Taste bud volumes on the cut side did not differ between cut-only and cut + diet rats ($P > 0.10$). Therefore, nerve section had the greatest effect on reducing taste bud volume on the regenerated side of the animal.

Numbers of innervating neurons

There was substantial variation in the number of neurons that innervated single taste buds in each group (see Fig. 3). For example, in controls the number of neurons that innervated single taste buds ranged from 1–14, with a mean number (±SEM) of 8.2 (±0.8) neurons/taste bud. Similarly, the number of neurons innervating taste buds in the diet-only group ranged from 3–18 (mean = 9.7 ± 0.7), and the number of neurons on the uncut side of the tongue in cut-only and cut + diet rats ranged from 5–16 (mean = 8.9 ± 0.9) and 4–18 (mean = 10.2 ± 1.0), respectively. None of the experimental means in which the chorda tympani remained intact differed significantly from controls ($P > 0.10$). By comparison, the number of neurons innervating single taste buds on the regenerated side of the tongue in cut-only and in cut + diet rats was significantly less than in controls (range = 0–7 and 1–6 and means = 3.2 ± 0.6 and 3.3 ± 0.4 $\mu m^3 \times 10^4$, respectively; $P < 0.00001$). Therefore, significantly fewer neurons innervated single taste buds following regeneration of the chorda tympani nerve compared to the unoperated side, irrespective of the dietary manipulation.

Relationship between taste bud size and innervation as a result of unilateral chorda tympani section

In control rats the number of geniculate ganglion neurons that innervated single taste buds predicted the size of the taste bud (Fig. 3A). That is, the larger the taste bud, the more geniculate ganglion neurons that innervated it.

TABLE 2. Numbers of Chorda Tympani Nerve Labeled Geniculate Ganglion Cells

		Cut only		Cut + Diet	
Controls (right side)	Diet only (right side)	Uncut (Left side)	Regenerated (Right side)	Uncut (Left side)	Regenerated (Right side)
481	491	552	465	482	414
479	482	379	272	554	365
532	481	492	464	451	388
467	476	506	446	417	376
468	503	483	364	557	503
441	—	522	364	419	356
547	—	—	—	—	—
488	**487**	**489**	**396**	**480**	**400**

Numbers of labeled cells for controls and diet-only rats are from ganglia in which the right chorda tympani nerve was labeled. Numbers of labeled cells in the next four columns are shown for the uncut (left) and regenerated (right) sides of cut-only and cut + diet rats. Bold numbers are means of labeled cells for each group.

TABLE 3. Summary of Diet and Nerve Cut Effects on Taste Bud Volume, the Relationship and Ratio of Taste Bud Volume to Innervating Ganglion Cells, and the Number of Innervating Ganglion Cells

	Effect compared to controls					
	Taste bud volume		Single papilla label correlation taste bud volume:innervating neurons		Geniculate ganglion counts	
Group	Uncut (Left side)	Sectioned (Right side)	Uncut (Left side)	Regenerated (Right side)	Uncut (Left side)	Regenerated (Right side)
Diet only	⇓		No correlation decreased ratio		=	
Cut only	⇓	⇓⇓	No correlation decreased ratio	No correlation increased ratio	=	⇓
Cut + diet	⇓	⇓⇓	No correlation decreased ratio	No correlation increased ratio	=	⇓

Single and double arrows pointing downward denote smaller taste buds than controls or significantly fewer geniculate ganglion cells than controls. Double arrows denote smaller taste buds than on the uncut side.

This provides a framework for which comparisons can be made following dietary and/or surgical manipulations.

In comparison to the high correlation between taste bud volume and numbers of innervating neurons in controls, there were no significant correlations for any of the five experimental comparisons. Remarkably, merely placing rats on a sodium-restricted diet at adulthood (i.e., diet only) led to a lack of the relationship between ganglion cell number and taste bud volume ($r = 0.38$; Fig. 3B). However, even though there was no overall correlation between ganglion cell numbers and taste bud volume, many of the individual values were within the 95% confidence interval generated from the control data (Fig. 3B). This suggests that the correct number of ganglion cells matched many, but not all, of the taste bud volumes. Similarly, there was no significant correlation for this relationship on the intact side of the tongue in cut-only and cut + diet rats ($r = 0.56$ and 0.29, respectively; $P > 0.10$; Fig. 3C,D). In both groups there were proportionately more ganglion cells innervating single taste buds than would be predicted from controls (Fig. 3A). The most dramatic disruption of the normal relationship, however, occurred in the taste buds in which the chorda tympani regenerated. The correlations of 0.34 and 0.26 for cut-only and cut + diet rats, respectively, were not significant ($P > 0.10$; Fig. 3C,D). Interestingly, in both groups there were some taste buds in which few (or no) ganglion cells were labeled even though the label in fungiform papillae met criterion and there was a taste bud and an apparent pore. Unlike the uncut, contralateral side, taste buds innervated by the regenerated chorda tympani nerve had fewer, not more, ganglion cells innervating them than predicted from controls (Fig. 3C,D). Therefore, intact taste buds (i.e., taste buds in which the chorda tympani was not sectioned) in diet-only, cut-only and diet + cut rats had a greater number of ganglion cells/taste bud volume as compared to controls. Taste buds on the regenerated side of the tongue displayed fewer mean labeled ganglion cells / taste bud volume as compared to controls (Figs 3C,D; $P < 0.0001$).

Total chorda tympani ganglion cell numbers

Approximately 50 days after unilateral chorda tympani nerve section, there was a reliable decrease in the number of geniculate ganglion cells that sent axonal processes into the regenerated nerve compared to those that sent processes into the uncut nerve (Table 2). No diet-related differences existed ($P > 0.10$); therefore, data were pooled for the regenerated and uncut sides for cut-only and cut + diet rats. Consequently, there were 484.5 ±16.9 ganglion cells on the intact side and 398.1 ±18.3 ganglion cells on the regenerated side (mean ± SEM; Table 2; $P < 0.02$). Furthermore, the number of ganglion cells on the intact side was indistinguishable from the number of geniculate ganglion cells in controls (487.9 ± 14.3; $P > 0.8$; Table 2).

DISCUSSION

Either instituting a sodium-restricted diet and/or unilaterally sectioning the chorda tympani nerve for 50–60 days prior to measurements disrupted the function whereby taste bud volume is matched by numbers of innervating geniculate ganglion cells. The function was disrupted in intact taste buds (i.e., those in which the chorda tympani nerve remained intact) primarily by decreased taste bud volume, and was disrupted in reinnervated taste buds primarily by decreased numbers of innervating ganglion cells. The present results demonstrate that 1) the neuron/target mismatch occurs in the adult peripheral gustatory system; 2) the effects occur on both the regenerated side of the tongue and the side distant to the

axotomy; 3) the mechanisms that underlie the mismatches differ, depending on the experimental manipulation; and 4) the anatomical effects are more widespread and are distinct from the functional changes. A summary of these findings is shown in Table 3.

Experimentally induced changes in taste bud volume

One of the factors that influences taste bud size is dietary sodium. This is best illustrated in diet-only rats in which taste bud volume was reduced significantly compared to controls. While the mechanism for the decreased volume is not yet known, it is likely that both the volume and numbers of individual taste bud cells were affected. Support for this comes from an analysis of taste bud cell numbers in rats fed a sodium-restricted diet throughout pre- and postnatal development. Cell-cycle kinetics were dramatically altered in these rats, resulting in a decreased mean number of taste bud cells and an apparent decreased volume of individual taste bud cells compared to controls (Hendricks et al., 2004). Such alterations in both cell-cycle kinetics and cell volume may be related to the growth-regulating roles of sodium (e.g., Hill, 1987; Roy-Clavel et al., 1999).

Another factor operating to affect taste bud size is the presence of a unilateral section of the chorda tympani nerve. In fact, taste bud volumes on the intact side of unilaterally sectioned rats (cut-only and cut+ diet rats) were significantly reduced. This is perhaps the most interesting and unexpected finding of this study. Axotomy-induced release of circulating factors (e.g., cytokines) may regulate some aspect(s) of cell cycle kinetics on the intact side through common circulatory and lymphatic systems (Hellekant, 1976; Maher, 1985). The cell cycle parameters potentially influenced by such factors include reduced rates of proliferation, migration of dividing cells into the taste bud, lifespan, and increased turnover rates. While cell cycle dynamics or analyses of axotomy-induced factors that may play such a role in altering individual taste bud cell kinetics have gone unexplored on both the regenerated and intact side of the tongue, the maintenance of whole taste buds (as compared to single taste bud cells) on the contralateral, intact side of the tongue is unaffected by nerve section (and dietary manipulation) (McCluskey and Hill, 2002). A future, extensive analyses of how taste bud volumes decrease in response to dietary sodium and to nerve section is warranted given the current results and would require a careful analysis of taste bud cell numbers, taste cell kinetics, and overall epithelial changes at various periods after nerve section.

Although there are clear and unexpected changes in taste bud volume on the intact side of the tongues of rats sustaining unilateral chorda tympani sections, the largest alterations in volumes were present in taste buds in which their respective nerve regenerated. In fact, taste buds on the regenerated side of the tongue in both cut-only and cut + diet rats were similarly reduced (Fig. 3C,D), indicating that the predominant factor in decreased taste bud size relates to the regenerated nerve and not to the dietary manipulation. We believe that this is due primarily to a decrease in the number of geniculate ganglion cells available to innervate single taste buds (see below).

Experimentally induced changes in geniculate ganglion cell numbers

Following a 50-day period after axotomy there is a substantial decrease (~20%) in the number of geniculate ganglion cells available to send processes into the chorda tympani nerve. The consistency of our results in that each of the 12 nerve-sectioned rats had fewer ganglion cells on the regenerated side of the animal leads us to believe that there was a significant and reliable loss of many neurons available to make connections with the regenerating target taste bud, regardless of the animal's dietary history. Such a loss translates into a mismatch between numbers of neurons innervating a taste bud and the taste bud's volume. This is consistent with the observation of an ~67% loss of myelinated neurons in hamsters receiving a complete crush injury (i.e., not axotomy) of the chorda tympani nerve (Cain et al., 1996). However, our data contrasts with a previous report in which axotomy failed to reliably eliminate ganglion cells (Whitehead et al., 1995). The discrepancy may be resolved by differences in methodologies and/or by species differences. Unlike the methods used here in which the nerve was bulk-labeled in rats and then cell counts made following imaging of entire ganglia, the earlier study labeled ganglion cells through fluorescent tracer injections in the hamster tongue (Whitehead et al., 1995). Furthermore, the loss of ganglion cells after axotomy in rat, but not in hamster, is consistent with a species-dependent difference in taste bud loss following gustatory nerve section. Specifically, hamster taste buds in fungiform papillae appear to have a greater degree of taste bud cell survival following denervation (Whitehead et al., 1987; Barry and Savoy, 1993; Ganchrow et al., 2003) that may relate to a maintenance of neurotrophins and their receptors in papillae following nerve section (Ganchrow et al., 2003). The same degree of taste bud survival after chorda tympani nerve section is not evident in rat (Hård af Segerstad et al., 1989). Therefore, the apparent increased susceptibility of rat taste buds to nerve section compared to that in hamsters may also be reflected in an increased susceptibility of geniculate ganglion neurons to axotomy-induced cell death.

Relationship between taste bud size and innervation

Compared to control adult rats, there is a lack of correlation between taste bud volume and the number of innervating geniculate ganglion neurons during early postnatal development (Krimm and Hill, 1998) and when rats were raised on a sodium-restricted diet (Krimm and Hill, 1999). In both cases, the lack of neuron/target match was attributed primarily to changes in taste bud volume and not to differences in numbers of ganglion cells. Specifically, during normal development taste buds grow to match the number of neurons innervating them over the first 40 days postnatal (Krimm and Hill, 1998), with the mature complement of ganglion cells innervating single taste buds determined before 10 days postnatal (Krimm and Hill, 2000). Similarly, developmental dietary sodium restriction decreases taste bud size, but not the mean number of ganglion cells innervating taste buds, resulting in a mismatch between neurons and their targets (Krimm and Hill, 1999). Restoration of dietary sodium at adulthood in developmentally sodium-restricted rats results in a match

between taste bud volume and ganglion cell number through growth of taste buds (Krimm and Hill, 1999).

In the current study, it is also the decreased taste bud volumes and not the number of innervating neurons that accounts for the neuron/target mismatch in diet only and on the intact side of the tongue in cut-only and cut + diet rats (Fig. 3B–D). Thus, "appropriate" numbers of innervating neurons neither support the normal size of taste buds nor do they rearrange to match the decreased size. As noted earlier, future experiments could determine if there is also a mismatch between innervating neurons and numbers of taste receptor cells by a detailed examination of the cellular effects located in the lingual epithelia.

By comparison, taste buds innervated by a regenerated chorda tympani nerve have both decreased taste bud volumes and decreased numbers of innervating geniculate ganglion cells (Fig. 3C,D). Interestingly, the decrease in both measures, especially in cut + diet rats, failed to lead to a corresponding correlation between the two variables. Since so many of the data points fall below the normal regression line, indicating fewer than predicted ganglion cell numbers, and since there are no apparent diet-related differences in the function on the regenerated side, we contend that the relationship between taste bud volume and innervation is disrupted primarily by decreased numbers of ganglion cells. This is reasonable, given the ~20% reduction in labeled chorda tympani neurons in the regenerated side in both cut-only and cut + diet rats. However, the numbers of regenerated neurons innervating single taste buds (see Fig. 3C,D) appear much less than a 20% reduction in the number of available neurons. This may be due in part to a less than full regeneration of available neurons at the level of taste buds. That is, 50 days after axotomy may be sufficient to restore taste responses in the regenerated chorda tympani (Hill and Phillips, 1994), but full neural regeneration may not be complete until much later. Although Cain et al. (1996) found a large, long-term decrease in the number of axons in the hamster chorda tympani nerve following nerve crush, they saw a significant increase in the number myelinated axons between the periods of 2–4 weeks and 5–16 weeks post nerve injury. Therefore, it is possible that labeling regenerated taste buds much longer than 7 weeks (~50 days) postsection in current experiments would lead to at least a partial restoration of the relationship between taste bud volume and number of innervating neurons. However, Montavon et al. (1996) showed that regenerated neurons have decreased branching at the taste bud in rats even as long as 6 months postsurgery, indicating that the decreased numbers of available ganglion neurons following axotomy may also have reduced numbers of processes available to innervate taste bud cells. These factors make the ratio of taste bud volume to number of innervating ganglion neurons even more surprising because current data show not only is there a mismatch but that relatively large taste buds can be supported by very few innervating neurons (Fig. 3C,D).

The mature peripheral gustatory system is presumably challenged to send an unchanging afferent message to the brain by way of single chorda tympani nerve fibers, even though taste receptor cells are continually turning over. This assumes that either single chorda tympani fibers find the appropriate functional set of receptor cells as new ones are born and/or nerve fibers dictate the functional characteristics of newly innervated receptor cells. Such a match probably involves both trophic and tropic factors. Indeed, Ninomiya and colleagues (Ninomiya, 1998; Yasumatsu et al., 2003) suggest that the nerve matches the functional properties on the taste receptor cells, indicating a possible influence of the nerve on the receptors. It is likely that neurotrophins (e.g., BDNF) that are maintained in fungiform papillae following nerve section (Ganchrow et al., 2003) and are important in early development of fungiform papillae and taste buds (Nosrat and Olson, 1995, 1998; Nosrat et al., 1996, 2000, 2001) play a role in how the nerve makes synaptic contacts with their target taste buds. The current findings may reflect complex alterations in factors regulating neuronal survival (e.g., on the regenerated side of the tongue) and maintenance of proper numbers of taste bud cells (e.g., on the contralateral, intact side of the tongue).

The dissociation of the neuron/target match may also result in alterations in the afferent gustatory signal sent to brainstem structures. More or less convergence of taste receptor cells on single afferent fibers coupled with possible changes in receptor cell transduction elements would lead to changing afferent messages during development, following early dietary sodium restriction, and, in the case of the current findings, following unilateral nerve section on both the intact and regenerated side of the tongue. Long-term recordings from single chorda tympani fibers would be instrumental in identifying and characterizing these potential changes. Although the single neuron response properties have not been examined, there are clear and profound functional alterations in whole-nerve taste responses.

Corresponding taste response alterations

The morphological alterations shown here are accompanied by functional taste response changes. However, unlike the structural alterations, only taste responses in sodium-restricted rats sustaining unilateral chorda tympani nerve section are altered. Relative taste responses from sodium-replete rats, regardless of nerve section, are normal (Hill and Phillips, 1994). Unilateral chorda tympani section in rats fed a sodium-restricted diet at adulthood results in a regenerated nerve that has selectively attenuated responses to sodium salts (Hill and Phillips, 1994). However, there are other, novel effects. In the same rat, the contralateral, uncut nerve is supersensitive to sodium salts. The supersensitivity progressively occurs over the first 40–50 days postsection, following an initial subnormal response. Moreover, these dynamic changes occur in the absence of reinnervation on the axotomized side of the tongue (Hill and Phillips, 1994), only when the sodium-restricted diet is instituted soon after axotomy (McCluskey and Hill, 2002), and when lingual damage (through axotomy of the chorda tympani, glossopharyngeal, or trigeminal nerves or through localized thermal injury) is coupled with sodium restriction (Hendricks et al., 2004).

Since the functional effects are specific to sodium salt taste responses and only occur when chorda tympani sectioning is coupled with dietary manipulations, it is unclear how the morphological alterations detailed in the current study relate to functional consequences in the periphery. For example, taste buds on the side of the tongue in which the chorda tympani nerve regenerated in sodium-replete rats have dramatic morphological changes

without corresponding stimulus-specific, functional deficits.

Taken together, the functional and morphological findings demonstrate that the peripheral gustatory system is especially plastic, even in adulthood. Sectioning a gustatory nerve or dietary sodium restriction leads to a profound alteration in the relationship between the nerve and its target through multiple mechanisms; both procedures result in functional as well as morphological alterations. As such, this system may be useful as a model system to examine the cellular and molecular events involved in nerve/target interactions in the peripheral nervous system.

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