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Taste Bud Cell Dynamics during Normal and Sodium-Restricted Development

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ABSTRACT

Taste bud volume increases over the postnatal period to match the number of neurons providing innervation. To clarify age-related changes in fungiform taste bud volume, the current study investigated developmental changes in taste bud cell number, proliferation rate, and life span. Taste bud growth can largely be accounted for by addition of cytokeratin-19-positive taste bud cells. Examination of taste bud cell kinetics with ^{3}H-thymidine autoradiography revealed that cell life span and turnover periods were not altered during normal development but that cells were produced more rapidly in young rats, a prominent modification that could lead to increased taste bud size. By comparison, dietary sodium restriction instituted during pre- and postnatal development results in small taste buds at adulthood as a result of fewer cytokeratin-19-positive cells. The dietary manipulation also had profound influences on taste bud growth kinetics, including an increased latency for cells to enter the taste bud and longer life span and turnover periods. These studies provide fundamental, new information about taste bud development under normal conditions and after environmental manipulations that impact nerve/target matching. J. Comp. Neurol. 472:173–182, 2004.

Indexing terms: taste bud; thymidine; cytokeratin; turnover; cell life span; receptor cell; development; sensory

Gustatory papillae and associated taste buds form in the rat fetus before or near term, but taste buds do not reach structural maturity until after postnatal day 30 (P30; Farbman, 1965; Mistretta, 1972; Mbiene and Farbman, 1993). The heterogeneous distribution of mature fungiform taste bud volume does not emerge until P40 (Krimm and Hill, 1998). By P40, fungiform taste bud size is proportional to the amount of innervation by chorda tympani neurons: The larger the taste bud, the more neurons that innervate it (Krimm and Hill, 1998). This relationship does not exist for taste buds in P10–30 rats, in which disproportionately large numbers of chorda tympani fibers innervate taste buds in comparison with their size (Krimm and Hill, 1998). It is the increase in taste bud size with age, and not neural rearrangement, that accounts for the neuron/target match (Krimm and Hill, 2000). Specifically, the number of neurons innervating taste buds at P10 can predict their mature size. Furthermore, most neurons that innervate a taste bud at P10 maintain contact through P40 (Krimm and Hill, 2000).

The match between fungiform taste bud volume and innervation number that exists in adulthood requires weeks of taste bud growth. Although it is clear that taste buds increase in size with age, it is not clear how the increase occurs. In the circumvallate papilla, age-related increases in taste bud volume during development are primarily a function of cell addition (Hosley and Oakley, 1987). One aim of the current work was to assess whether similar changes occur in fungiform papilla by using antibodies directed against cytokeratin-19 (CK-19), a marker for differentiated taste bud cells (Zhang et al., 1995).

Although there has been some attention directed toward how taste buds increase in volume, little work has exam-

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ined the kinetics by which cells are added. Indeed, the taste system presents interesting problems for studies of cell life span. For example, progenitor cells, located at the base and margins of taste buds (e.g., Beidler and Smallman, 1965), supply not only taste buds but nongustatory cells within papillae. Alterations either in the rate of production of new cells or in the proportion of cells ultimately residing in taste buds could lead to increases in taste bud size. Furthermore, taste bud cells are periodically renewed; in adults, the turnover period is approximately 10 days (Beidler and Smallman, 1965; Farbman, 1980). Increased taste bud size could, therefore, also occur if the rate of turnover is low in young rats. That is, the increase in size may relate to age-related differences in cell death. Clearly, multiple processes could account for the overall increase in taste bud volume with age. To address these problems, we examined the life span kinetics of taste bud cells following a single injection of tritiated thymidine.

The age-dependent increase in taste bud volume can be manipulated experimentally. By simply restricting dietary sodium from early conception throughout the life of the offspring, the growth of taste buds in rats is "frozen" at a small, immature state until dietary sodium is restored (Krimm and Hill, 1999). As noted for normal development, there is no apparent change in innervation. Rather, the nerve/target mismatch is due primarily to an arrested growth of taste buds. Thus, parallel studies comparing the potential addition of taste bud cells and the life span kinetics of taste bud cells in sodium-restricted rats with those of normally developing rats provide further insights into the processes involved in taste bud development. Accordingly, we examined three groups: neonatal and adult rats raised on a sodium-replete diet and adult rats raised on a sodium-restricted diet.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (Harlan, Dublin, VA) housed on a constant 12:12-hour light/dark schedule (lights on at 0700) were used. All experiments were endorsed by the Animal Care and Use Committee of the University of Virginia and followed guidelines set out by the National Institutes of Health. Every attempt was made to minimize the number of animals required.

Dietary regimens

Sodium-replete diet. Three dams and one male were housed together for 14 days. At the conclusion of this period, the male was removed and the dams were housed individually. Dams were checked for litters at 0800 and 1800 hr daily. Litters were culled to 10 on the day after birth and were weaned at 21 days. Rats had free access to standard Purina rat chow (1% NaCl) and water. Pups remained with their mother until weaning at P21.

Sodium-restricted diet. Harems consisting of three experienced female breeders and one male were housed together for 5 days, during which time the rats were fed normal chow and water. On day 6, females were removed to separate cages and placed on a diet of low-sodium chow (0.03% NaCl; ICN Biochemicals, Aurora, OH) and distilled water. Cages were checked daily for births at 0800 and 1800 hr. Animals were maintained on the low-sodium diet and are referred to as *sodium-restricted* rats.

Experiment 1: do taste buds grow by adding new cells?

CK-19 immunolabeling. After an overdose with urethane, tongues from neonatal (P10), adult (P60), and sodium-restricted (P60) rats were excised and flash frozen in –30°C isopentane. Serial 20-μm coronal cryostat sections were taken from the tip and midregions of the tongue defined by Krimm and Hill (1998), thaw mounted on slides, and immunohistochemically processed for visualization of the cytoskeletal marker CK-19. Sections were postfixed in 4% paraformaldehyde for 3 minutes and incubated in primary antisera directed against CK-19 (1:400; Sigma, St. Louis, MO; mixed in 4% normal goat serum and 0.2% Triton X, all in 0.1 M PBS, pH 7.4) overnight. Slides were incubated in a secondary antibody for 1 hour (tetramethylrhodamine-conjugated goat anti-mouse; 1:400; Jackson Immunochemical; mixed in 0.2% Triton X in 0.1M PBS, pH 7.4). The green fluorescent nuclear marker Sytox (1 μM in distilled water for 30 seconds; Molecular Probes, Portland,OR) was used to visualize all cell nuclei. All steps were preceded by three 3-minute rinses of 0.1 M PBS (pH 7.4), except for Sytox staining, which was preceded with similar rinses of distilled water. Slides were allowed to air dry and then coverslipped using Pro-Long antifade mounting medium (Molecular Probes).

Cell counts and taste bud volume measurements. Optical sections of 1 μm were obtained throughout the entire extent of taste buds by using a Nikon PCM200 confocal laser scanning microscope with Simple PCI software (Compix Imaging Systems, Cranberry Township, PA). Image stacks were imported into Adobe Photoshop as TIF files for counting of CK-19-immunopositive cells. By using a transparent layer in Photoshop, Sytox-stained nuclei that were completely encircled by CK-19 immunoreactivity were outlined and counted as labeled cells. The overlay was then placed over the next 1-μm section, and only new nuclei fitting the previous criterion were outlined and counted. Because entire taste buds were reconstructed, all CK-19-positive cells were counted, overcoming the need for stereological sampling procedures (Coggeshall and Lekan, 1996; Popken and Farel, 1997).

Taste bud volume measurements were accomplished by tracing sections of taste buds with a computer microscope (Neurolucida, Colchester, VT). The boundaries of taste buds were defined as the extent of CK-19-immunopositive labeling. Because CK-19 immunoreactivity is specific to taste bud cells in lingual epithelia, the peripheral germinal cells typically associated with buds were not included. This resulted in lower taste bud volume estimates than those previously reported (Whitehead et al., 1985; Krimm and Hill, 1998).

Taste bud cell counts and volumes were obtained from a total of 35–45 taste buds/group (from at least three nonlittermates/group). To reduce potential sources of error, the experimenter was blind to the experimental condition, and all cell counts were performed independently of taste bud volume measurements. Differences in taste bud cell numbers and CK-19 label volume among neonates, adults, and sodium-restricted rats were assessed via ANOVA, with Tukey's post hoc analysis applied where appropriate.

Experiment 2: taste bud cell kinetics

^{3}H-thymidine labeling. Neonate (P10), adult (P40), and sodium-restricted (P40) rats received a single dose of ^{3}H-thymidine (i.p., 5.0 μCi/g body weight, specific activity 72 Ci/mM) at 9 PM, the time of greatest proliferation in adults (pilot data, and Farbman, 1980). This is considered as time = 0 (t_0). Three rats (no more than two littermates per group) were subsequently killed at 9 AM at 1.5, 3.5, 6.5, 8.5, 10.5, 12.5, 14.5, and 16.5 days postinjection. Rats were deeply anesthetized with urethane and perfused transcardially with Krebs solution, followed by 4% paraformaldehyde. Tongues were removed, embedded in paraffin, sectioned at 10 μm, and mounted on gelatin-subbed slides. Slides were deparaffinized, dipped in NTB-2 emulsion (diluted 1:1 with distilled water; Kodak, Rochester, NY), and processed according to standard emulsion autoradiographic techniques (Cleaver, 1967; Brunjes et al., 1989). After an exposure period optimized through pilot experiments, slides were developed with D-19 developer and Kodafix fixer (Kodak), counterstained with methylene blue, and coverslipped.

Tissue analysis. Sixty taste buds were examined at each postinjection period. The experimenter was blind to group condition and the period postinjection during data collection. Because postmitotic taste bud cells (CK-19$^+$; Zhang et al., 1995) are primarily located within the central core of the taste bud, we used this region for data collection. The centermost section through each taste bud was determined as defined by the presence of a taste pore and its area measured with an image-analysis system (MCID; Image Research, St. Catherines, Ontario, Canada; Whitehead et al., 1985; Whitehead and Kachele, 1994; Krimm and Hill, 1998). Taste buds were divided into zones, "lateral" and "central" to differentiate between likely nontaste and taste cells, respectively (see Fig 1A). The longest base-to-apex measurement (height) was taken. Additionally, a line perpendicular to this base-to-apex measurement was extended to cross the greatest extent of the taste bud (width). Taste bud width and height were divided into quarters, with two most lateral and two central zones (Fig. 1A).

Within these boundaries, the numbers of labeled cells were counted and plotted for each time point with the MCID system. A cell was considered "labeled" if the number of silver grains over its nucleus was at least 3 SD above background. Background was calculated as the number of grains in an equivalent area on the slide that did not contain cell nuclei. Background estimates were obtained from at least 20 separate locations on each slide to calibrate for potential differences in slide processing.

Data analysis. Multiple variables were assessed, including 1) the time when the peak number of labeled ^{3}H-thymidine cells was observed, 2) the average time for new cells to enter the taste bud (t_i; defined as the time on the ascending portion of the cell accumulation curve when 50% of the labeled cells entered the taste bud), 3) the average taste bud cell life span (t_2; defined as the point on the descending portion of the curve when 50% of the cell remained), and 4) the turnover period ($t^2 - t^1$; the time for which cells resided within the central compartment of the taste bud). These measures were determined from a total of 480 taste buds per age group. Average time to enter the taste bud and average life span of taste bud cells were calculated as by Beidler and Smallman (1965) and Farbman (1980).

Differences in taste bud cell number between adults and neonates and differences between adults and sodium-restricted rats were assessed by ANOVA, with Tukey's post hoc analysis where appropriate. Distribution and number of cells within the taste bud at discrete time points were tabulated and assessed using ANOVA and Tukey's post hoc analysis where appropriate. Photoshop (Adobe Systems, San Jose, CA) was used to compose digital images. Only contrast and brightness were enhanced in the images.

RESULTS

CK-19 expression

Expression of CK-19 was robustly distributed throughout the central regions of neonatal and adult taste buds regardless of dietary condition (Fig. 1B–1D). Cells surrounding the margins of the taste bud were CK-19 negative. CK-19-positive cells had a typical fusiform shape, extending from the base of the taste bud to the pore (Fig. 1B–D). In the section containing the taste pore, CK-19-positive nuclei were crowded throughout the lower half of the taste bud (especially in large taste buds). CK-19-positive cells also often demonstrated a basal process that extended into the nuclei free region at the base of the taste bud (Fig. 1B–D). In agreement with previous studies, few if any CK-19-negative cells were observed within the boundaries of CK-19-positive cells (Hosley and Oakley, 1987). Indeed, among 125 taste buds sampled (containing a total of 3,488 cells), fewer than 10 CK-19-negative nuclei were interspersed within CK-19-positive regions.

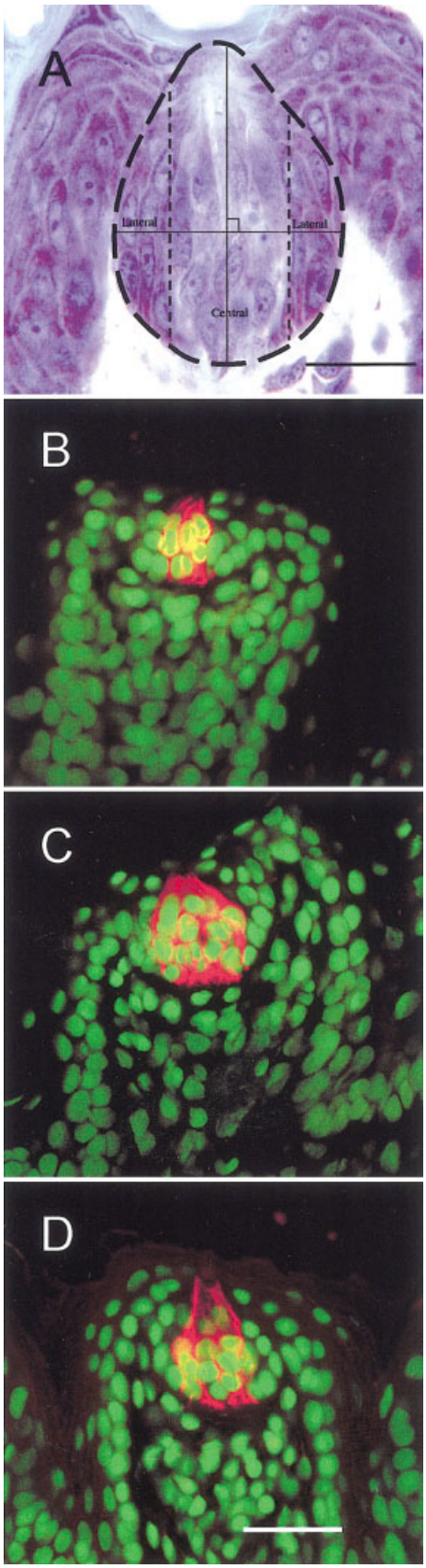
Taste bud cell numbers increase during development

There was more than a fourfold increase in the number of CK-19-positive cells within the fungiform taste bud during development [$F(2,122) = 127.7$, $P < 0.0001$; Fig. 1B,C]. Neonatal taste buds (n = 46) contained an average of 12.9 ± 0.6 (SEM) CK-19-positive cells per taste bud (Fig. 2A). The average number of immunopositive cells in adult taste buds (n = 42) was greater (49.2 ± 2.2), and the cells were more heterogeneously distributed than in neonates, ranging from 35 to 109 cells (Fig. 2A). Taste buds located in the tips of adult tongues contained numbers of cells similar to those located in the midregion (Fig. 3), indicating that the reported regional differences in taste bud volume (Krimm and Hill, 1998) may be due to differences in individual cell volume and/or in the number of perigemmal cells and not to differences in taste bud cell numbers.

Mean taste bud volume, as defined by the region inhabited by CK-19-positive cells, increased dramatically with age, from 2,218.5 ± 134 μm^3 in neonatal rats to 9,611.1 ± 591 μm^3 in adults [$F(2,122) = 84.4$, $P < 0.001$; Fig. 2B]. The smaller volume found in younger animals is consistent with the finding that they contained fewer immunopositive cells. Values observed in adults were similar to those described by Krimm and Hill (1998).

Taste bud cell numbers following developmental sodium restriction

Taste buds in sodium-restricted rats contained significantly fewer CK-19-positive taste bud cells compared with



age-matched diet controls [Fig. 1C,D; n = 37, mean = 22.4 ± 2.0; $F(2,122) = 127.7$, $P < 0.0001$]. However, unlike taste buds in neonates, taste buds in sodium-restricted rats had a greater range of CK-19-positive taste bud cells per taste bud (Fig. 2A). The distribution of labeled cells/taste bud in sodium-restricted rats was similar to that in adults even though the average number of CK-19-positive cells was approximately 50% of that in controls. Taste bud volume in sodium-restricted rats was likewise smaller compared with that in age-matched controls (4,724.4 ± 430 μm^3 compared with 9,611.1 ± 591 μm^3) but significantly greater than in neonates (Fig. 2B).

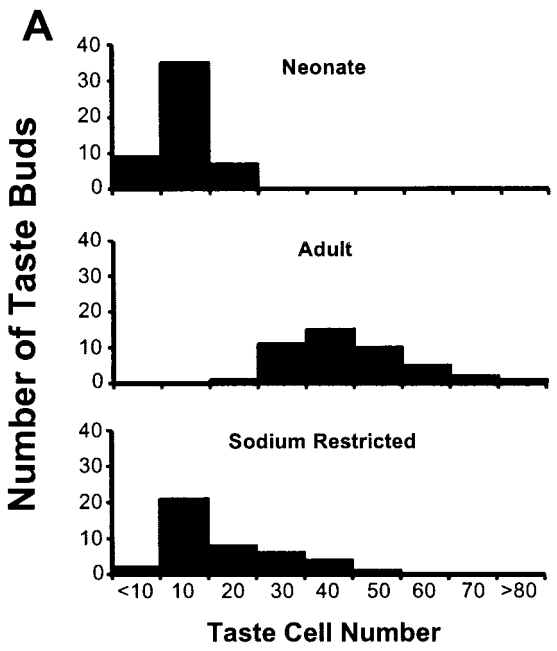
Developmental changes in taste cell production

All labeled taste bud cells. In adults, large numbers of densely labeled cells were observed in the most lateral regions of taste buds 1.5 days after ^{3}H-thymidine injection, with few cells observed in central zones (Figs. 4A, 5A). With longer survival periods, increasing numbers of labeled cells were observed in central regions, indicating that cell progeny had migrated into the core of taste buds. Peak numbers of labeled cells were observed in the central region 8.5 days following injection (3.7 ± 0.2; Figs. 4D, 5A). Subsequently, numbers of labeled cells in the central area decreased sharply, reaching a nadir at 12.5 days (Figs. 4G, 5A). An increase in labeled cells in the central region of taste buds reemerged after 12.5 days to reach levels near 50% of peak label at 16.5 days (Fig. 5A).

In neonates, similar numbers of labeled cells were observed at the margins (lateral regions) of the taste bud at 1.5 days postinjection (3.5 ± 0.2 and 3.9 ± 0.5 for neonates and adults, respectively; $P = 0.41$; Fig. 4A,B). In the central compartment, labeled cells were significantly less numerous than adults at that time [$F(2,179) = 11.1$, $P > 0.0001$; Fig. 5B]. Once again, with longer survival times, increasing numbers of labeled cells accumulated in the central compartment. The peak number of labeled cells was observed 6.5 days postinjection, when on average 4.9 ± 0.2 labeled cells per taste bud were encountered (Figs. 4E, 5B), a number significantly higher than that seen at the time of peak labeling (at 8.5 days postinjection) in adult taste buds [$F(2,179) = 6.2$, $P = 0.003$; Figs. 4H, 5B].

Entry into the taste bud. As noted earlier, the time from thymidine injection (t_0) to the point when 50% of the peak number of cells entered the central region of the taste bud (t_1) is an estimate of the time required for the processes of basal cell division, progeny migration, and cell differentiation (Beidler and Smallman, 1965; Cleaver, 1967; Farbman, 1980). The time taken for cells to enter the taste bud was substantially longer in neonate than in adult rats (2.7 vs. 1.8 days; Fig. 5A,B).

Fig. 1. **A:** Photomicrograph of a coronal section (2 μm; plastic) through the center of a fungiform taste bud. The taste bud divisions "lateral" and "central" were created to differentiate between presumptive nontaste and taste cells, respectively. The maximal width was divided into quarters, with the two lateralmost quarters designated as "lateral" and the two central areas denoted as "central." **B–D:** Photomicrographs of fluorescently labeled taste buds in neonatal (B), adult (C), and sodium-restricted (D) rats. CK-19-like labeling is shown in red and the nuclear stain, Sytox, is shown in green. Scale bar = 50 μm in A; 30 μm in D (applies to B–D).



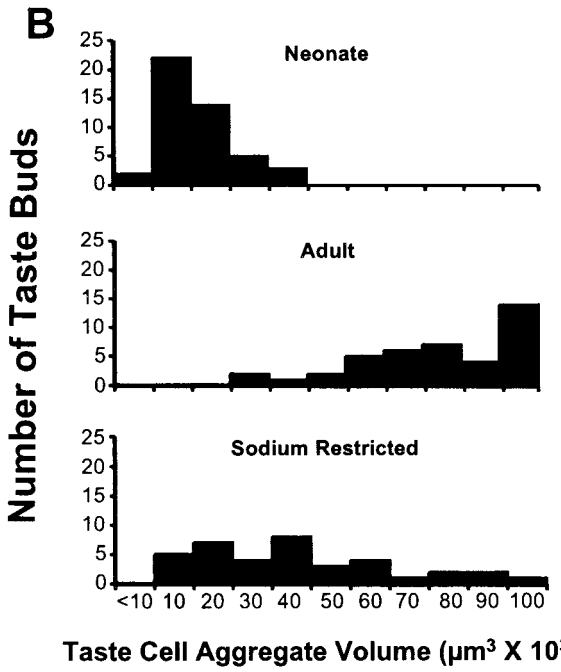


Fig. 2. Frequency distributions of taste cell number (**A**) and taste cell aggregate volume (**B**) for neonatal (top), adult (middle), and sodium-restricted (bottom) rats.

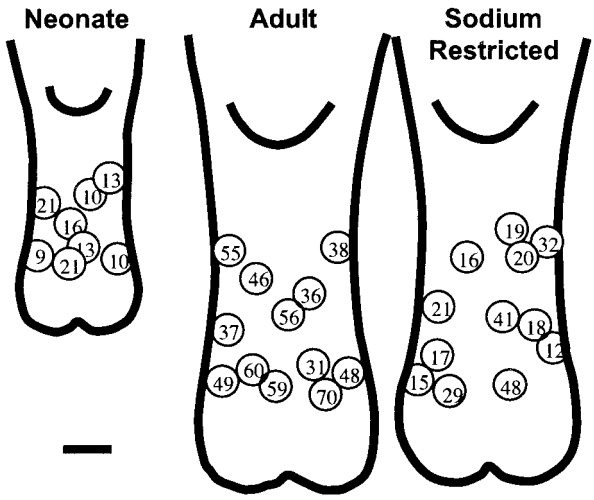


Fig. 3. Location of fungiform taste buds imaged from neonatal (left), adult (center), and sodium-restricted (right) rats. Numbers refer to the total cell numbers for the respective taste bud. Scale bar = 1.5 mm.

Presumptive taste bud cell life span and turnover period. Since taste bud cells are postmitotic, the average life span can be estimated by the period from t_0 until the half-maximal peak value on the descending portion of the population curve (t_2). In agreement with Beidler and Smallman's (1965) estimate of 258 ± 50 hours (10.8 ± 2.1 days), the average life span of taste bud cells in adults was 11.6 days (Fig. 5A). Based on analyses of all labeled cells, the average life span of neonates was considerably longer (16.5 days) than that in adults (Fig. 5B). However, in neonates, there was a decline in labeled cells in the central taste bud region following the peak labeling at 6.5 days. The average number of labeled cells did not decrease to a half-maximal value before ascending again to a second peak (Fig. 5B). Thus, subsequent divisions appeared to increase the number of labeled cells away from the requisite 50th percentile (see below under First-generation taste bud cells).

The "turnover period" is an indication of the time for which a cell resides within the central region of the taste bud. Accordingly, the difference between the average life span (t_2) and the time to enter the taste bud (t_1) was calculated for neonate and adult rats. The turnover period of taste bud cells was significantly longer in neonates compared with adults (13.8 days compared with 9.8 days, respectively), primarily because of their significantly longer life span.

Effects of sodium restriction. Similar numbers of labeled cells were observed at the margins of the taste bud at 1.5 days postinjection in sodium-restricted and adult rats (4.2 ± 0.3 vs. 3.9 ± 0.5; $P = 0.413$; Fig. 4A,C). However, the apparent movement of labeled cells into the taste bud in sodium-restricted rats was not as rapid as that in controls. Populations of labeled cells within the taste bud required 3.8 days to enter the central region, which was substantially longer than in controls (1.8 days; Fig. 5A,C). Thereafter, a much different pattern emerged in sodium-restricted rats, including an explosive rate of addition of cells in the center of the taste bud, between 3.5 and 6.5 days postinjection (Fig. 5C). Peak labeling was delayed, with maximal levels not reached until 14.5 days (Fig. 4C,I,F), when the average number of labeled cells within the taste bud reached 5.5 ± 0.3 cells, a value significantly greater than that seen in controls [3.7 ± 0.2 cells; $F(2,179) = 6.2$, $P = 0.003$; Fig. 5C]. The length of time for which the average taste bud cell resided within the taste bud was 16.4 days in sodium-restricted rats, and

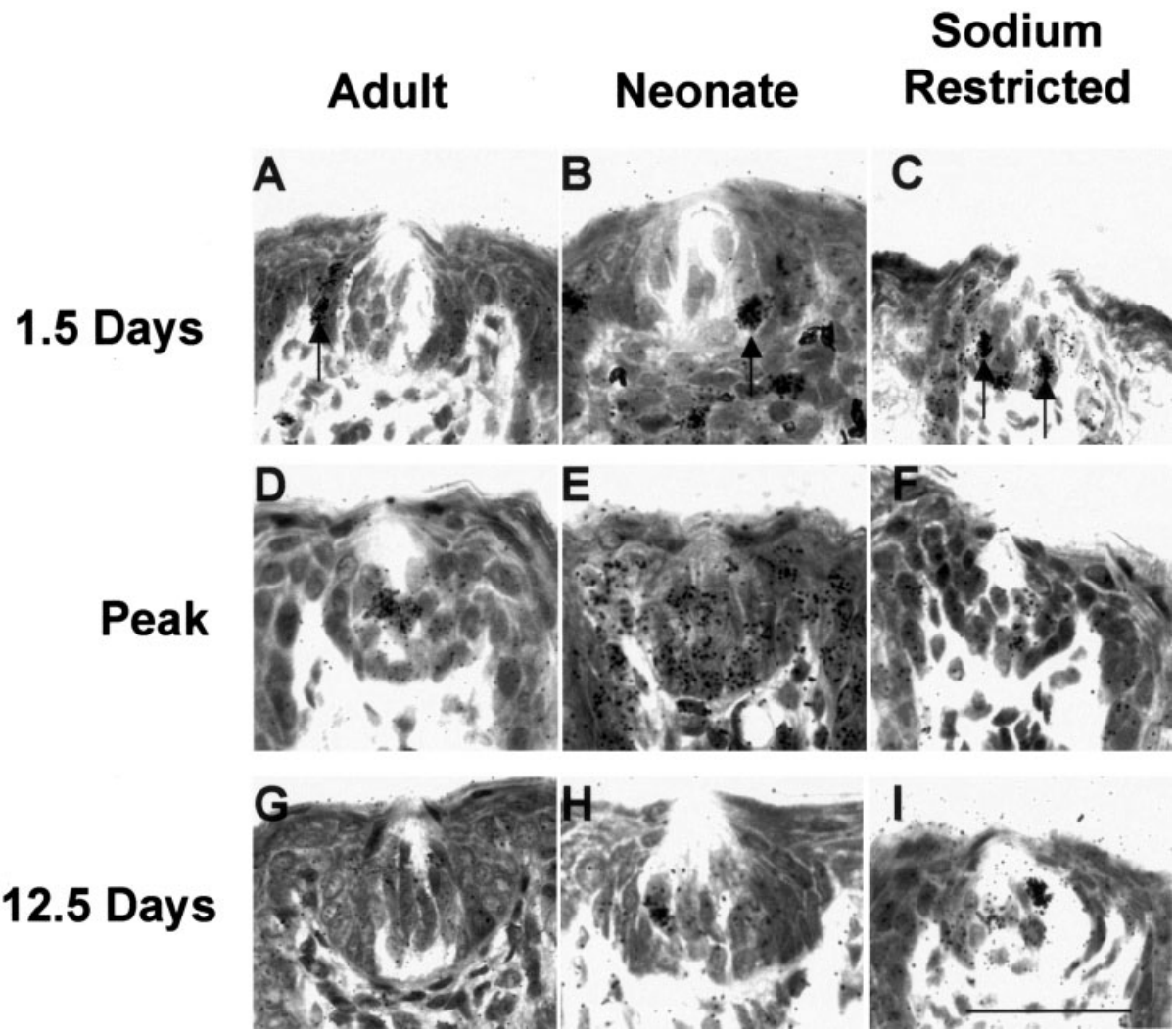


Fig. 4. Coronal sections through the pore region of adult (**A,D,G**), neonatal (**B,E,H**), and sodium-restricted (**C,F,I**) rats following a single pulse of ^{3}H-thymidine. Taste buds are shown for 1.5 days postinjection (A–C), at the peak of labeling (D, 8.5 days postinjection; E, 6.5 days postinjection; F, 6.5 days postinjection), and at 12.5 days postinjection (G,H,I). Arrows in A–C point to cells heavily labeled with ^{3}H-thymidine. Scale bar = 50 μm.

the average cell turnover was 12.6 days, both values significantly greater than those for adult controls (11.6 and 9.8 days for life span and turnover periods, respectively; Fig. 5A,C).

First-generation taste bud cells. The data presented above were based on the entire population of labeled cells and thus undoubtedly included data for cells from precursors that had undergone multiple rounds of mitotic division. Indeed, Figure 5 demonstrates that multiple cell divisions were represented, which likely influenced measures of life span and turnover. Unlike methods based on thymidine analogs, such bromodeoxyuridine, ^{3}H-thymidine can be used to differentiate among various generations of cells produced from one injection, because the number of silver grains observed is roughly proportional to the amount of label within the cell (Cleaver, 1967; Rakic, 1977). As progenitor cells reenter the cell cycle, the available pool of labeled thymidine incorporated in DNA is progressively reduced. Therefore, the most heavily labeled cells represent the first generation. We quantified the number of silver grains over cell nuclei at the earliest survival time, 1.5 days, to establish an estimate of the labeling characteristics of the first generation. Although the average cell cycle time has not been identified for taste buds, even in adults, we chose to use the 1.5-day period here because the average cell cycle time in rodent tongue epithelium (Potten et al., 2002) and in many other tissues (Cleaver, 1967) is 20–30 hours. In this study, we assumed the simplest situation, with precursors undergoing asymmetrical division to produce one taste bud cell and one cell that reverts to the mitotic cycle. Accordingly, a frequency distribution of grain counts over cell nuclei was plotted, and the density of silver grains in the upper 50th percentile of the distribution was then used as a conservative criterion to define first-generation cells operationally.

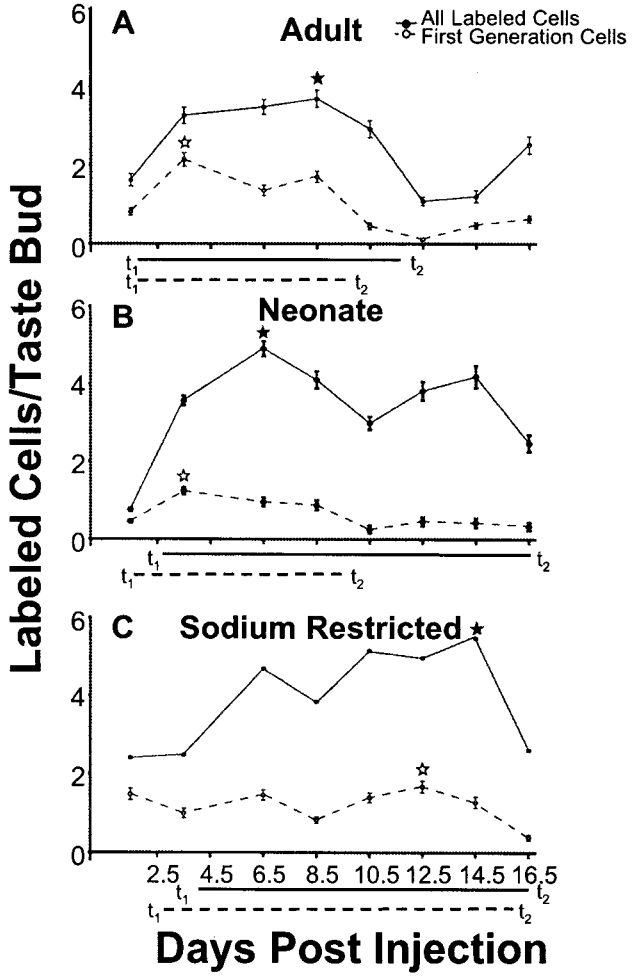


Fig. 5. Number of ^{3}H-thymidine labeled taste bud cells plotted for each period after injection. Solid symbols with solid lines indicate cells that had at least the criterion number of silver grains over background (all labeled cells). Dashed lines indicate the subset of heavily labeled cells within the total labeled population (first-generation cells). Turnover periods for all cells (solid line) and for first-generation cells are shown below each graph. Average time for cells to enter taste buds is denoted as t_1 and average taste bud cell life span is denoted as t_2 (see text for explanations of calculations). Peak labeling is denoted for all labeled cells and for first-generation cells by solid and open stars, respectively. **A:** In adults, data from all labeled cells were mirrored by data from first-generation cells. **B:** Neonatal life span calculations derived from all cells had two peaks over time, which were not reflected in first-generation cells. **C:** Taste buds in sodium-restricted rats had an increased number of cells over a longer period of time than in either adults or neonates.

Normal development. In examining first-generation cells, average time to enter the taste bud in adults was very similar to the estimated period obtained from analyses of all labeled presumptive taste bud cells (1.9 and 1.8 days, respectively; Fig. 5A). First-generation progeny also largely contributed to life span calculations in adult rats, reducing the average life span to 9.5 days (a net change of 2 days). Thus, the turnover period for presumptive taste bud cells was 7.6 days in adults (Fig. 5A).

In startling contrast, taste bud growth parameters in neonates were altered. With only first-generation cells used to recalculate taste bud cell life span, the second peak was sharply attenuated (Fig. 5B, dotted line compared with solid line). The corrected neonate taste bud cell life span was 9.3 days. Average time to enter the taste bud was also altered, reducing the average time to enter the taste bud to 1.9 days, a value close to that calculated for adult rats. Because of the alterations in both average period of entry and average life span, the turnover period was 7.4 days.

Sodium restriction. The taste bud kinetics generated from first-division cells were similar to values generated from all cell divisions in sodium-restricted rats (Fig. 5C; dotted line compared with solid line). Heavily labeled cells were strongly represented at 1.5 days following injection, with 60% of cells within the taste bud reaching criterion. This value was higher than that obtained at 3.5 days. However, if the value for average time to enter is extrapolated from the slope between 3.5 and 6.5 days (the interval for average period to enter for all cells), average time to enter is reduced to 2.6 days, producing a net decrease of approximately 29 hours compared with the case when all labeled cells were analyzed.

Once again, alterations in both average times of entry and average life span changed estimates of cell life span in sodium-restricted rats to 15.5 days and the turnover period to 12.9 days (Fig. 5C). The observation that these estimations were not radically different indicates that the first-generation cells contributed primarily to the calculation for life span in this measure. The taste bud cell kinetic data for all three groups, using total cells and first-generation cells, are summarized in Table 1.

DISCUSSION

During postnatal development, taste buds in rat fungiform papilla grow to match the number of innervating neurons. The current work has provided the first normative data characterizing this maturational process. Taste buds in mature rats displayed a wide diversity in overall volume, which was also reflected by the number of CK-19-positive cells within each taste bud. In contrast, taste buds from immature sodium-replete and from mature rats

TABLE 1. Taste Bud Cell Kinetics in Adult, Neonatal, and Sodium-Restricted Rats

	Time to enter (days)	Peak number	Time at peak (days)	Average life span (days)	Turnover period (days)
All cells					
Adult	1.8	3.7 ± 0.2	8.5	11.6	9.8
Neonate	2.7	4.9 ± 0.2	6.5	16.5	13.8
Sodium-restricted	3.8	5.5 ± 0.3	14.5	16.4	12.6
First generation					
Adult	1.9	2.1 ± 0.2	3.5	9.5	7.6
Neonate	1.9	1.2 ± 0.1	3.5	9.3	7.3
Sodium-restricted	2.6	1.7 ± 0.2	12.5	15.8	12.9

raised on a sodium-restricted diet contained far fewer CK-19-positive cells, which was mirrored by smaller volumes. Quantifying growth kinetics with injections of ^{3}H-thymidine was used to assess the manner by which this growth occurs. Cell proliferation rates of taste bud cells were much higher in young rats, whereas cell life span and turnover period remained constant, indicating that mitotic rate was probably the most important factor in taste bud growth. Dietary sodium restriction instituted during pre- and postnatal development had profound influences on taste bud growth kinetics, including lengthened taste bud cell production, life span, and turnover periods compared with neonate or adult control rats.

Normal development

Developmental increases in taste bud volume could occur via alterations in cell production, migration, maturation and/or death. Each of these alternatives is examined in turn below. Taste bud cells are produced by the division of basal cells found at the margins of taste buds. Altering the process by 1) increasing the total number of progenitor cells dividing (a variable known as the "growth fraction"; Cleaver, 1967) or 2) changing the rate at which they undergo mitosis might affect taste bud volume. Results presented in Figure 5 do not support the first hypothesis: Fewer labeled cells were observed 1.5 days postthymidine injection in neonatal rats than in adults. However, results consistent with the second alternative were observed in the rapid dilution of ^{3}H-thymidine label.

The germinal population producing taste bud cells also yields progeny that move into the general epithelia of the papilla (Beidler and Smallman, 1965). Increasing the relative proportion of daughter cells migrating into taste buds could ultimately increase cell number and taste bud size. The proportion of progenitor cells contributing to taste buds is not known, and factors controlling division and migration of cells have not been identified. However, neurally derived factors or factors that affect neural precursors may influence this process (Krimm and Hill, 1998; Nag and Wadhwa, 1999; Kusakabe et al., 2002).

With maturation, taste cells differentiate and develop the characteristic fusiform shape, and some taste bud cells make functional connections with innervating nerve fibers within the taste bud. The present findings (see, e.g., Table 1, Fig. 5) demonstrate that neither taste bud cell life span nor taste bud cell turnover accounts for the developmental increase in taste bud volume.

We found the mean taste bud cell life span in mature (and young) rats to be approximately 10 days, as did Beidler and Smallman (1965). Taste buds in circumvallate papillae exhibit a similar life span (Farbman, 1980), suggesting that it is a uniform feature of taste bud cells.

Possible mechanism for taste bud growth

Figure 2 indicates that CK-19-immunopositive cells are added as taste buds grow. The autoradiographic results suggest a possible mechanism for cell addition. Although the number of labeled cells 1.5 days after thymidine injection was lower in neonate than in adult animals (suggesting a smaller growth fraction), by 1 week the number of positive cells surpassed adult values. The difference was not due to the proportion of cells entering the taste bud from each division or rate of migration; indeed, the time and rate of entry of taste cells into the immature taste bud were equal to or greater than those seen in mature taste buds (23.1 vs. 26.6 hours, respectively). However, when the total number of all labeled cells was analyzed, neonatal taste bud cells did not demonstrate a sharp nadir (Fig. 5B). The lack of a deep trough between cycles seen in adults may indicate an influx of subsequent divisions of the progenitor population. Because the second rise of labeled cells in the central region of neonatal taste buds occurs more quickly than in adult taste buds, we propose that cell production is accelerated during postnatal development. One way to reconcile the disparate information—lower growth fraction with equal or lower rates of cell addition, turnover, and life span—is to have the progenitor population in neonatal animals dividing faster than that in adults (Fig. 6). This one parameter determines the overall addition of fungiform taste bud cells with age. Precisely how this developmental change occurs demands further clarification of the identity and behavior of the cells forming the progenitor population. The change could be derived from a simple decrease in the number of available precursor cells or, alternatively, be the result a more complicated regulation of multiple cell classes (e.g., populations of transiently amplifying cells).

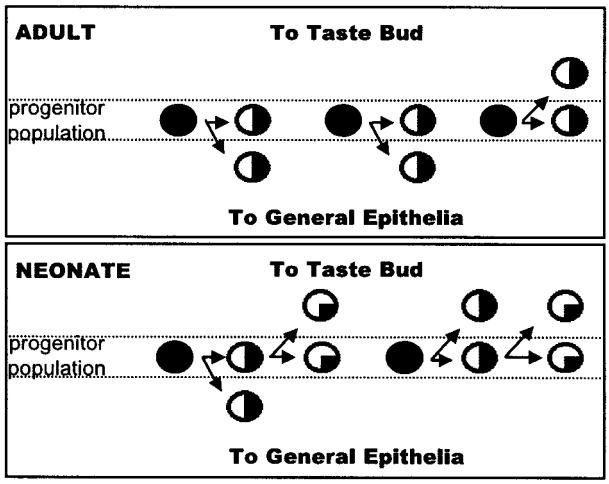


Fig. 6. Potential mechanism for developmental taste bud volume increase. Compared with taste buds in adults, taste buds in neonatal rats have fewer dividing cells per taste bud. If progenitor cells for taste buds in neonatal rats reenter the cell cycle at a greater frequency than in adults, taste bud cell number would increase over time. The proportion of cells migrating toward the taste bud may also increase during development to affect an increase in taste cell number.

Changes in cell cycle kinetics during development are a common means by which cell numbers can vary (Gong and Shipley, 1995; Miyama et al., 1997; Smith and Luskin, 1998; Caviness et al., 1999; Nowakowski et al., 2002). Among the many mechanisms that regulate epithelial tissue kinetics, neuron–target interactions during development play an important role in determining the proper number of cells in a structure. For example, postembryonic proliferation of myonuclei that form *Manduca sexta* leg muscles is dependent on innervation (Consoulas and Levine, 1997; Bayline et al., 2001). Innervation also has a dynamic role in governing proliferation of keratinocytes. After denervation, skin epithelial thickness is reduced within 72 hours, because of changes in proliferation, but

not differentiation or apoptosis, of keratinocytes (Huang et al., 1999). The role of innervation in maintaining taste buds parallels in part the role of innervation in skin epithelial thickness. Loss of innervation results in a loss or prominent structural alteration of taste buds (see, e.g., Guth, 1957; Fujimoto and Murray, 1970; Cheal and Oakley, 1977; Whitehead et al., 1987). However, unlike the case for skin epithelia, no change in proliferation rates occurs following denervation of fungiform taste buds in hamster (Oliver and Whitehead, 1992). Furthermore, significant levels of neurotrophins (brain-derived neurotrophic factor) are maintained in denervated taste tissue (Ganchrow et al., 2003). Neurotrophins and other growth factors often participate in regulation of epithelial tissue kinetics (Nag and Wadhwa, 1999).

Factors such as neurotrophins, neuropeptides, and electrical signals speed progression through the cell cycle by either 1) shortening G_1 or shortening the transition from G_1 to S or 2) increasing the growth fraction (Hansel et al., 2001; Lukaszewicz et al., 2002). For example, thalamic neurons release chemical signals, including basic fibroblast growth factor, to recruit neocortical precursors to divide faster (Cleaver, 1967; Schneider et al., 1991; Reznikov and van der Kooy, 1995; Dehay et al., 2001). In light of the dynamic role of innervation on target proliferation, it is possible that taste bud growth kinetics may be influenced by chorda tympani neurons. Basal cells at the margins of the immature taste bud may receive signals from neurons within the taste bud. As the taste bud nears mature size, the requirement for rapid cell addition would abate. Once taste buds mature, maintenance of proper taste bud size would require proliferation rates coordinated with taste bud cell death (Zeng et al., 1999; Haung and Lu, 2001). These coordinated processes might be at equilibrium once taste bud size matches numbers of innervating neurons (Krimm and Hill, 1998).

Sodium-restricted development

Dietary sodium restriction throughout pre- and postnatal development has profound effects on multiple aspects of taste bud cell kinetics, including time for newly formed cells to enter the taste bud, time at which peak numbers are observed, and turnover period (Table 1). Insofar as understanding the ultrastructure of the taste bud has been useful in examining the kinetics of circumvallate taste bud, the strategy might also help to elucidate the effects of sodium restriction. Farbman (1980) determined that turnover kinetics are contingent upon taste bud cell type. The three major cell types within the taste bud, dark cells (or type I) and light cells (type II and type III), differ in ultrastructural appearance and their dependence on innervation (Farbman, 1969, 1980; Pumplin et al., 1997; Yee et al., 2001). Dark cells, which have a life span of close to 9 days, outnumber light cells within the adult control taste bud by 2:1 (Farbman, 1969). Light cells (classified as type II cells) have protracted life spans and a slower rise time in numbers of cells labeled compared with dark cells (Farbman, 1980). Accordingly, alteration of the ratio of dark (type I) to light (type II and possibly type III) cells within the taste bud would alter the overall average taste bud cell kinetics. Perhaps populations of taste bud cells in sodium-restricted rats have similar kinetic characteristics, as do the light cells in the circumvallate taste buds of normal rats.

As mentioned above, taste bud cells are produced from multiple stem cell progenitors scattered around the perimeter of the taste bud (Stone et al., 1995, 2002). It is unknown whether there are separate progenitors for light and dark cells. Dietary sodium restriction may change circulating or local factors in the tongue and thus alter taste bud cell production patterns and life span. The idea that the local environment may affect the peripheral gustatory system in a dramatic, but not permanent, manner is intriguing. Indeed, nerve-dependent processes relating to function and structure of the peripheral gustatory system are altered profoundly through the introduction of a low-sodium diet (Hill et al., 1986; Hill, 1987; Hill and Przekop, 1988; Ye et al., 1993; Krimm and Hill, 1998). It is possible, therefore, that taste bud cell kinetics should also be affected, as seen here.

In conclusion, the continual normative renewal of taste bud cells throughout development and adulthood in the taste bud provides a useful model system with which to study epithelial renewal, neuron/target matching, and other systems-level mechanisms. A more detailed focus on progenitor cell identification as well as elucidation of the factors controlling cell proliferation at the taste bud would yield important information. For example, the current experiments may provide an important background for further studies examining the role that neurotrophins play in regulating taste bud size by altering taste bud cell kinetics. Additional experimental manipulations that include dietary variables may help to unmask such factors in the future.

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