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The Timing of α-Gustducin Expression during Cell Renewal in Rat Vallate Taste Buds

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Abstract

The G protein subunit α-gustducin is expressed in a subset of light (Type II) but not in dark (Type I) cells in rat vallate taste buds. The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) is incorporated into DNA during the S-phase of the cell cycle and can be used to determine the time of origin of a cell. In this study, 31 rats were injected with BrdU (50 mg/kg i.p.) and perfused at various times, from 2.5 to 10.5 days, following BrdU administration. Vallate papillae were embedded in polyester wax, cut into 4 μm transverse sections, and characterized with antibodies to BrdU and α-gustducin. Sections were processed for indirect immunofluorescence or with an immunoperoxidase procedure. From immunoperoxidase material on 21 rats, counts of α-gustducin- and BrdU-labeled cells were obtained from 300–800 taste bud profiles at each survival time; a total of 4122 taste bud profiles were examined. Cells with nuclei immunoreactive for BrdU occurred within the taste buds at 2.5 days and double-labeled cells were clearly evident at 3.5 days; a small number of double-labeled cells were seen as early as 2.5 days. Double-labeled cells reached a peak at 6.5 days and did not decline significantly by 10.5 days. Cells labeled for BrdU but not α-gustducin peaked at 5.5 days and showed a significant decline by 8.5 days. These latter cells included light cells not expressing α-gustducin and dark cells, which have previously been shown to have a shorter life span than light cells. These data suggest that expression of α-gustducin appears very early in a cell's life span and that these cells are longer lived than many of the cells that do not express this G protein.

Introduction

Gustducin is an α-subunit of a G protein which has been cloned from taste bud-bearing tissue and which is closely related to the transducins (McLaughlin *et al.*, 1992). Behavioral and electrophysiological studies on gustducin knockout mice suggest that α-gustducin is involved in the transduction of both sweet- and bitter-tasting stimuli (Wong *et al.*, 1996). A role for gustducin in sweet and bitter taste is also supported by the differential expression of gustducin across subpopulations of taste buds (Boughter *et al.*, 1997). That is, in taste buds that are relatively more responsive to sweet (palate) or bitter (vallate and foliate papillae) stimuli there are significantly more gustducin-expressing cells than in those less responsive (fungiform papillae) to these compounds. Electron-microscopic observations suggest that gustducin is expressed by light (Type II) cells in the rat vallate papilla (Tabata *et al.*, 1995; Menco *et al.*, 1997). At the light microscopic level, cells expressing α-gustducin have the morphological characteristics of a mature light cell: a spindle shape, round in cross section, with both apical and basal processes (Boughter *et al.*, 1997). A number of human blood group antigens (A, B, H and Lewis[b]) are also expressed by rat taste bud cells (Smith *et al.*, 1994). Of these, the A blood group is restricted to light cells, but these are not all immunoreactive for α-gustducin (Pumplin *et al.*, 1996), suggesting that α-gustducin is expressed in a subset of light cells, not in the entire population.

The cells within a taste bud undergo continual turnover, with a life span of ~10 days in rat fungiform (Beidler and Smallman, 1965) and vallate (Farbman, 1980) papillae. Based on studies following [^{3}H]thymidine incorporation during cell division, dark (Type I) cells in the rat vallate papilla have a life span of ~9 days (Farbman, 1980). Although it could not be accurately measured, light (Type II) cells appear to have a longer life span, suggesting that they are a cell type distinct from the dark cells. On the other hand, similar experiments on the mouse vallate papilla concluded, on the basis of the timing of [^{3}H]thymidine incorporation into different cell types, that taste bud cells progressed from basal cells to dark cells, through an

intermediate stage, to light cells (Delay *et al.*, 1986). Thus, taste bud cells arise either from different lineages (Farbman, 1980) or from only a single lineage in which cells differentiate from dark cells to light cells via intermediate cells (Delay *et al.*, 1986). Mosaic analysis suggests that taste bud cells arise from local epithelium and that there is more than one cell lineage within the taste bud (Stone *et al.*, 1995).

5-Bromo-2′-deoxyuridine (BrdU) is a thymidine analogue that is incorporated during the S-phase of the cell cycle. Single-stranded DNA containing BrdU can be detected immunohistochemically (Miller and Nowakowski, 1988). Immunohistochemical detection of BrdU has been used for the analysis of the proliferative characteristics of glioma cells (Shibui *et al.*, 1989) and cells in the developing CNS (Miller and Nowakowski, 1988), and for the study of cell turnover in mouse tongue epithelium (Hume, 1989). In the present study, the timing of the expression of α-gustducin in taste cells of the rat vallate papilla during cell differentiation was investigated using antibodies against α-gustducin and BrdU at various time periods after BrdU incorporation. If α-gustducin is a critical component in the transduction of sweet and/or bitter stimuli, the first appearance of this G protein would set a limit on how soon during differentiation a taste receptor cell could possibly respond to gustatory stimulation.

Materials and methods

Tissue preparation

Adult male rats, maintained on a 12/12 light/dark cycle (7:00 a.m. to 7:00 p.m. in the light) and weighing 260–400 g, were injected (50 mg/kg i.p.) with BrdU (Boehringer Mannheim, Indianapolis, IN). This concentration of BrdU results in labeling of proliferating cortical neurons identical to that seen with [^{3}H]thymidine and is not toxic, even when applied to neonatal rats (Miller and Nowakowski, 1988) or developing mice (Nowakowski *et al.*, 1989). The BrdU injections were made at 9:00 p.m., which is the time when the greatest number of cells are expected to be in S-phase (Farbman, 1980). Thirty-one rats were killed at daily intervals, ranging from 2.5 days following BrdU administration to 6.5 days, and at 8.5 and 10.5 days. Two additional rats were used for control experiments; these animals were killed 5.5 days after BrdU injection. Counts were made on taste buds from 21 rats, which were processed for immunoperoxidase labeling; tissue from 10 animals processed for immunofluorescence was observed on the confocal microscope, but was not quantified (see below).

The rats were anesthetized with xylazine (0.2 mg/kg i.p.) and ketamine hydrochloride (2.5 mg/kg i.p.), and killed at 10:00 a.m. on the assigned day. The rats were perfused transcardially, first with phosphate-buffered saline (PBS; pH 7.4) until all the blood was removed and then with cold, freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the portion of the tongue containing the vallate papilla was excised and immersed in the same fixative for 1.5 h at 4°C. The tongue region was cut coronally into 400 μm sections with a vibratome. These sections were viewed under a dissecting microscope to select those containing a vallate trench. The chosen sections were dehydrated through a graded alcohol series, then embedded in a polyester wax designed to melt at 37°C (Electron Microscopy Sciences, Fort Washington, PA). After cooling to –20°C, the embedded tissue was sectioned (4 μm) on a rotary microtome parallel to the trench and transversely to the long axis of the taste buds. Sections were mounted on slides coated with poly-L-lysine; data were taken from every fouth section, which was separated by at least 12 μm from the previous one. The slides were dried at 37°C overnight and then dehydrated with 100% alcohol, which removed the polyester wax, before further processing.

Antibodies and immunohistochemistry

The sections were treated with 2 M HCl at 37°C for 1 h to denature the double-stranded DNA and then with 0.1 M boric acid (adjusted to pH 8.5 with NaOH) to neutralize the acidity of HCl. Sections were preincubated with a solution of 1% bovine serum albumin and 10% normal goat serum in PBS for 1 h and then incubated with the mixture of primary antibodies for 1 h. Primary antibodies included a mouse monoclonal IgG against BrdU (anti-BrdU, 2.5 μg/ml; Boehringer Mannheim) and a rabbit polyclonal IgG against α-gustducin [$G_{\alpha\text{-gust}}$ (I-20), 1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA]. Anti-BrdU specifically binds to BrdU and cross-reacts with iodouridine (10%), but not with fluorodeoxyuridine nor with any endogenous cellular components such as thymidine or uridine (Boehringer Mannheim). $G_{\alpha\text{-gust}}$ (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 G_α subunits, including rod or cone α-transducin (Santa Cruz Biotechnology). After incubation with primary antibodies, the sections were treated with either fluorescent secondary antibodies or with an avidin–biotin immunoperoxidase procedure.

Immunofluorescence

Immunoreacted sections were rinsed in PBS and then reacted with a mixture of fluorescein-conjugated goat anti-mouse IgG (FITC, 10 μl/ml; Molecular Probes, Eugene, OR) and rhodamine-conjugated goat anti-rabbit IgG (Texas Red, 10 μl/ml; Molecular Probes) for 1 h at room temperature. The stained sections were rinsed in PBS and covered with non-fading medium (Fluoromount-G; Southern Biotechnology Associates, Birmingham, AL). BrdU immunoreactivity was seen as a green reaction product and gustducin immunoreactivity as red.

Immunoperoxidase

Immunoreacted sections were reacted with a secondary biotinylated goat anti-mouse IgG (10 μg/ml; Vector

Laboratories, Burlingame, CA) for 10 min and then with avidin and biotin (Vectastain ABC Elite) for 5 min. Diaminobenzidine–nickel chloride (0.05% DAB + 0.1% $NiCl_2$ in Tris buffer; Sigma Chemical Co., St Louis, MO) with H_2O_2 (0.025%, Sigma) was used as a chromogen for the BrdU reaction. The immunoreacted complex of antibodies to BrdU produced a black to blue-black reaction product. Any reactivity of the avidin from the first reaction was blocked by biotin (100 μl/ml; Vector Laboratories) before application of the remaining reagents. Biotinylated goat anti-rabbit IgG (10 μg/ml; Vector Laboratories) was applied as a secondary antibody and DAB without $NiCl_2$ was used as a chromogen in the second processing. The gustducin label was observed as a brown reaction product. After dehydration of the immunostained sections through a graded alcohol series and HemoD (Fisher Scientific, Pittsburgh, PA), the sections were coverslipped with Permaslip (Alban Scientific, St Louis, MO). Unless otherwise stated, reactions were conducted at room temperature. The sections were washed with PBS three times for 5 min each before every step.

Control experiments used the protocol described, but with the primary antibodies omitted. Omission of the primary antibodies resulted in loss of specific staining. In previous experiments on rat vallate taste buds, we preincubated the tissue with α-gustducin control peptide (3.7 μM; Santa Cruz Biotechnology) for 2 h at room temperature, which effectively blocked all immunoreactivity to the α-gustducin antibody (Boughter *et al.*, 1997).

Confocal and conventional microscopy

Immunofluorescence of labeled taste bud cells was photographed with a 63×/1.4 NA lens on a Zeiss confocal laser-scanning microscope (CLSM). Using the CLSM, optical sections were acquired at intervals of 1 or 3 μm, then combined into stereo images and/or viewed successively to observe cells. To examine overlap of antibody labeling, two stacks of optical sections were taken in quick succession starting at the same specimen depth. One stack was obtained from the fluorescein channel (BrdU) and the other stack from the rhodamine channel (α-gustducin). Confocal images were stored on Zip disks for analysis off-line. For visual identification of overlap, the stacks of rhodamine and fluorescein images were each combined into split-stereo images at 6° tilt, using software provided with the confocal microscope. The split-stereo images, or a pair of corresponding images taken at the same depth in each stack, were rendered in red and green, respectively, then superimposed to obtain the final colored image. Double-labeling was readily determined because the BrdU signal was superimposed over the nucleus and the α-gustducin signal was confined to the cytoplasm. Brightness and contrast were adjusted by normal procedures on the CLSM or with Photoshop (Adobe Systems, San Jose, CA), and the photomicrographs were printed on a dye-sublimation printer (Fujix Pictrography 3000; Fuji Photo Film U.S.A., Elmsford, NY).

Taste cells processed with the immunoperoxidase method were observed on a Nikon Microphot and counted at a magnification of 1250× or 2500×, using a plan 100×/1.25 NA oil-immersion objective. Images of all the taste buds in each section were captured at a lower magnification (250×) and printed on a Codonics VP-3500 videoprinter (Codonics, Middleburgh Heights, OH) so that each taste bud could be labeled and cataloged. Once this procedure was completed for all 4122 taste buds, each was observed at high magnification and the labeled cells in each taste bud were counted. Photographs for publication were acquired using a Polaroid DMC digital camera (Polaroid, Cambridge, MA). Brightness, contrast and color balance were adjusted by the DMC acquisition program and Photoshop prior to printing on the Fujix printer.

Cell counting

Immunofluorescent material was used to observe the presence of BrdU and α-gustducin at several time points after BrdU administration, but these cells were not counted because it was difficult to identify the boundaries of the taste buds in this material and many BrdU-labeled cells lay outside these boundaries, especially at shorter survival times. Taste cells processed with the immunoperoxidase method were counted at a magnification of 1250×. Gustducin-expressing cells were counted when the section contained both the nucleus and labeled cytoplasm around the nucleus. Cells with the cytoplasm stained but without a visible nucleus were not counted. BrdU label was observed as a mottled or punctate reaction product or as solid staining within a nucleus. A cell with punctate reaction product in the nucleus was counted when the size of the reaction product was at least 2 μm long and 1 μm wide; this criterion was chosen because it was difficult to discriminate smaller portions of reaction product from background noise. When the nucleus was completely stained it was sometimes difficult to differentiate the α-gustducin reaction product in the cytoplasm around it, which was often quite thin. In that case, the cell was observed at 2500× to confirm cytoplasmic staining.

Precautions were taken not to count a taste cell twice. The average length of a vallate taste cell nucleus is ~10 μm (Boughter *et al.*, 1997). Each side of the vallate trench on both sides of each vallate papilla was sectioned into 4-μm-thick sections. The sections from which data were acquired were separated by at least 12 μm, ensuring that no cell was counted more than once. Every section that could be included from each animal that met this criterion was included (some sections were lost or damaged during processing), resulting in 1–4 sections per vallate trench. The counts of labeled cells were the number of BrdU- and α-gustducin-labeled cells per taste bud profile in each section. Therefore, some sections were undoubtedly cut

through the same taste buds but because of the 12 μm separation could not have included the same cell nuclei. The final counts of labeled cells are expressed as labeled cells per taste bud profile, with no implication that this represents an estimate of the number of cells per taste bud. Counting only cells with visible nuclei in the section grossly underestimates the number of cells per taste bud, but is necessary since BrdU is only incorporated into the nucleus of the cell.

Statistical analysis

We used a one-way ANOVA to compare the numbers of labeled cells across days; *post hoc* comparisons between any two days were made using the Scheffé test for multiple comparisons (SPSS for Windows, v. 6.1; SPSS, Inc., Chicago, IL).

Results

In an initial experiment, immunofluorescent material was generated from 10 rats, ranging from 2.5 to 6.5 days after BrdU injection. From this material it was clear that a few α-gustducin-expressing cells were double-labeled with BrdU as early as 3.5 days after BrdU administration (Figure 1A, arrow) and that there were more double-labeled cells at 6.5 days (Figure 1B, arrows). However, even though it was quite easy to determine the occurrence of double labeling in these immunofluorescent sections, we chose not to quantify these data for two reasons. First, it was impossible to determine the boundaries of each taste bud profile unambiguously. Since BrdU-labeled nuclei often occurred outside the taste buds, especially at the shorter survival times, this limited our ability to accurately count the number of labeled cells occurring within each taste bud profile. Second, because there were so many taste buds on each section, we were concerned there would be fading of the fluorescence during the course of trying to quantify this material. For these reasons, we conducted a second experiment on an additional 21 rats using the immunoperoxidase reaction.

In the immunoperoxidase-labeled material, immunoreactivity for BrdU was seen as a black to blue-black reaction product within a nucleus. Sometimes the entire nucleus contained reaction product and at other times patches were scattered within the nucleus. Immunoreactivity for α-gustducin was seen in this material as a brown reaction product. Examples of taste buds containing cells immunoreactive for BrdU and/or α-gustducin are shown in Figure 1C–F. These images, photographed at 1250× using an oil-immersion lens, represent material from rats at 3.5 (C), 6.5 (D), 8.5 (E) and 10.5 (F) days following BrdU injection. In these 4 μm sections the boundaries of the taste buds are evident even in tissue that is not counterstained. Cells that were double-labeled for BrdU and α-gustducin are indicated by arrows and cells within the taste buds labeled only for BrdU are indicated with asterisks. BrdU label was also seen in the nuclei of cells in the epithelium outside the taste buds. Immunoreactivity for α-gustducin was evident in many cells that were not also labeled for BrdU, including many that were sectioned above or below their nucleus. These latter cells appeared as small round or oval cells within the taste buds (Figure 1). Only those cells exhibiting a nucleus within the section were counted. Cells that had BrdU label in the nucleus and α-gustducin label in the cytoplasm were counted as double-labeled cells. In cases where a nucleus was stained heavily, it was sometimes difficult to decide whether the cytoplasm around the nucleus was also stained. In those cases, the cell was counted as double-labeled only if the cell was seen to have brown staining in the cytoplasm distinguished from the black staining in the nucleus at twice the magnification (2500×).

Almost all α-gustducin-labeled taste cells showed a circular-to-oval cross-sectional shape, which is a characteristic of light (Type II) cells. Some taste buds which were oriented longitudinally near the edges of the taste bud-bearing epithelium contained spindle-shaped α-gustducin-labeled cells with both apical and basal processes, which is also a morphological feature of light cells (see also Boughter *et al.*, 1997).

A summary of the cell counts is given in Table 1, which also shows the number of rats and the number of taste bud profiles that comprised the data obtained from the immunoperoxidase material. Counts are given as means (± SEM). The mean number of α-gustducin-labeled cells per taste bud profile did not vary systematically with survival time (Table 1, α-gustducin), although there was a significant main effect across days [one-way ANOVA, $F(6,4115) = 3.99$, $P < 0.001$]. However, *post hoc* comparisons showed that the number of α-gustducin-labeled cells differed significantly only between 4.5 and 10.5 days (Scheffé test, $P < 0.05$); no other comparisons were significant. The overall mean number of gustducin-labeled cells per taste bud profile was 2.22 (± 0.02).

BrdU-labeled cells were evident within the taste buds at 2.5 days and reached their peak at 5.5 days after BrdU injection (Table 1, Total BrdU). There was a significant effect of survival time on the number of BrdU-labeled cells [one-way ANOVA, $F(6,4115) = 12.05$, $P < 0.0001$]. The means at 4.5, 5.5 and 6.5 days were significantly higher than those at 2.5 and 3.5 days, whereas the means at 8.5 and 10.5 days were different from those at 2.5, but not 3.5–6.5 days (Scheffe test, $P < 0.05$). If only BrdU-labeled cells that were not immunoreactive for α-gustducin are considered (BrdU w/o α-gustducin), there is also a main effect of time [one-way ANOVA, $F(6,4115) = 9.42$, $P < 0.0001$], but the means at 8.5 and 10.5 days were significantly less than those at 5.5 days (Scheffé test, $P < 0.05$), showing a significant decline after reaching a peak.

There was a significant difference in the numbers of double-labeled (BrdU + α-gustducin) cells per taste bud profile across survival times [one-way ANOVA, $F(6,4115) = 21.74$, $P < 0.0001$]. The numbers at 6.5 and 8.5 days differed

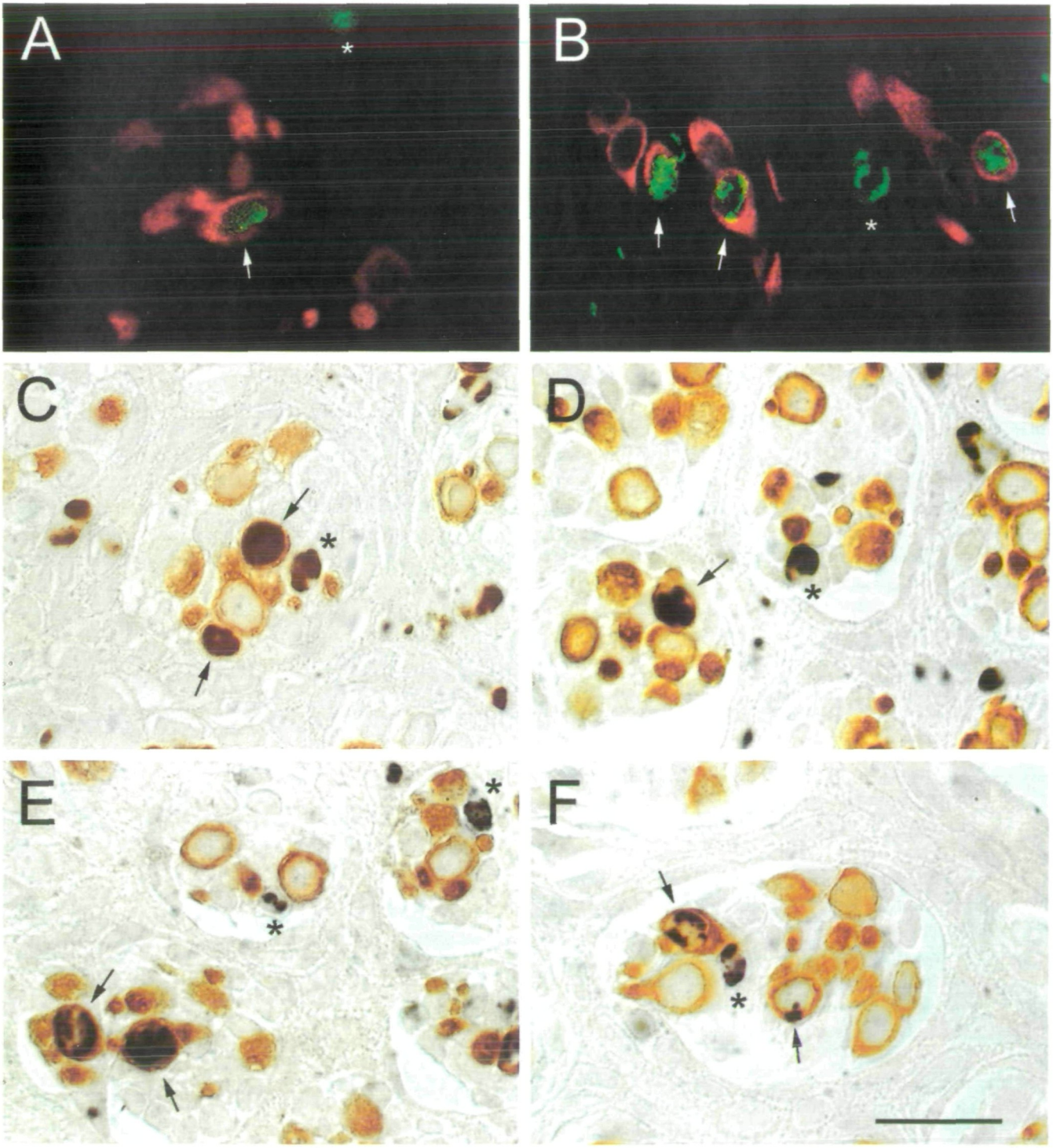

Figure 1 Photomicrographs of transverse 4 μm sections through rat vallate taste buds in material processed for immunofluorescence **(A, B)** and immunoperoxidase **(C–F)**. Arrows point to double-labeled cells; asterisks are adjacent to BrdU-labeled nuclei within taste buds whose cytoplasm is not also immunoreactive for α-gustducin. (A) Section from a rat 3.5 days after BrdU administration. (B) Immunofluorescent labeling in a section taken 6.5 days following BrdU. (C) Immunoperoxidase-labeled section 3.5 days after BrdU. (D–F) Sections from rats surviving 6.5 (D), 8.5 (E) and 10.5 (F) days after BrdU administration. Calibration bar = 20 μm.

significantly from those at 2.5, 3.5, 4.5 and 5.5 days (Scheffé test, $P < 0.05$). The number at 10.5 days was also different from the numbers at 2.5 and 3.5 days, but not from those at 4.5–8.5 days. The number of double-labeled cells/taste bud profile increased gradually until 5.5 days (0.10 ± 0.02) and was then doubled at 6.5 days (0.20 ± 0.02). This number

Table 1 Mean (± SEM) counts of labeled cells per taste bud profile in rat vallate taste buds processed for α-gustducin and BrdU immunoreactivity

Days	No. of rats	No. of taste bud profiles	α-gustducin	Total BrdU	BrdU alone	BrdU + α-gustducin
2.5	3	534	2.25 ± 0.06	0.67 ± 0.06	0.66 ± 0.06	0.01 ± 0.01
3.5	2	407	2.11 ± 0.06	0.74 ± 0.06	0.68 ± 0.06	0.06 ± 0.01
4.5	3	697	2.09 ± 0.06	1.09 ± 0.06	1.00 ± 0.05	0.09 ± 0.01
5.5	3	317	2.21 ± 0.08	1.26 ± 0.09	1.16 ± 0.09	0.10 ± 0.02
6.5	3	760	2.22 ± 0.06	1.17 ± 0.05	0.96 ± 0.04	0.21 ± 0.02
8.5	3	596	2.15 ± 0.07	1.00 ± 0.05	0.80 ± 0.05	0.20 ± 0.02
10.5	4	811	2.42 ± 0.05	0.96 ± 0.04	0.81 ± 0.04	0.15 ± 0.02
Total	21	4122				

decreased to 0.15 (± 0.02) at 10.5 days after the peak at 6.5 days, but these two values were not significantly different (Scheffé test, $P < 0.05$). Although there were only four double-labeled cells in 534 taste bud profiles (0.008 ± 0.005) at 2.5 days, it is clear that gustducin-expressing taste cells can first appear as early as 2.5 days after BrdU injection. The examples shown in Figure 1, which were chosen to illustrate double-labeled cells, would suggest that there are more double-labeled cells than those expressing only BrdU, but this was clearly not the case. The counts (Table 1) show that many more cells were BrdU-positive and α-gustducin-negative than were double-labeled.

Discussion

Several characteristics of the BrdU reaction product seen in the present experiment have been previously reported in studies using BrdU to study proliferating neurons (e.g. Miller and Nowakowski, 1988). First, the use of HCl to denature the DNA often resulted in expansion of the reaction product into the perikaryon (Figure 1B,D,F). In addition, there was variation in the distribution of the BrdU reaction product. Sometimes the reaction product was homogeneous and completely filled the nucleus (Figure 1C). In other instances, many of the BrdU-immunoreactive cells had a mottled or punctate reaction product (Figure 1F). These cells probably entered or left the S-phase during the time that BrdU was available, making them less able to incorporate as much of the BrdU as cells that were in the S-phase during the entire time that BrdU was available (Miller and Nowakowski, 1988). Nevertheless, by establishing a minimum criterion for what constituted a labeled nucleus, we feel confident that we included all of the BrdU-labeled cells in the 4122 taste bud profiles that were quantified in the present experiment.

At least two types of cells have been described in mammalian taste buds: dark (Type I) cells and light (Type II) cells (Farbman, 1965; Murray, 1971, 1973). In the rat vallate papilla, light cells are relatively large, have an electron-lucent cytoplasm and are circular-to-oval when cross-sectioned, whereas dark cells are smaller and have an electron-dense cytoplasm and an irregular outline that includes cytoplasmic projections lying between adjacent light cells (Pumplin *et al.*, 1997). Some investigators describe an intermediate cell in mouse vallate taste buds, which has characteristics between dark and light cells (Kinnamon *et al.*, 1985; Delay *et al.*, 1986). However, the multivariate distribution of a constellation of morphological characteristics suggests strongly that there are only two distinct cell types (light and dark) within rat vallate taste buds (Pumplin *et al.*, 1997). These cell types are generally characterized on the basis of morphological features at the electron-microscopic level, although the characteristic round-to-oval shape and lack of cytoplasmic projections makes the identification of light cells in the rat vallate papilla possible at the light-microscopic level, especially when the taste buds are cut transversely to their long axis (Pumplin *et al.*, 1997).

Labeled light cells have been observed in rat vallate taste papilla as early as 1.5 days after [^{3}H]thymidine injection and reach their peak at 6.5 days (Farbman, 1980). In the present study, α-gustducin-expressing cells were first observed at 2.5 days and reached their maximum number at 6.5 days (Table 1). In rat vallate taste buds, α-gustducin-expressing cells have been described as spindle-shaped, with round, thin processes extending toward the taste pore (Boughter *et al.*, 1997). These characteristics are morphological features of light (Type II) cells that are readily apparent in transverse sections. Cells in the rat vallate papilla begin to express α-gustducin relatively early in their life span, at least within 3.5 days of BrdU injection; a few double-labeled cells were evident at 2.5 days (Table 1). This early expression of α-gustducin was observed in rat vallate taste cells which already have the morphological features of mature light cells; even at very short survival times cells expressing this G protein did not resemble dark cells. Electron-microscopic studies have also confirmed that α-gustducin is expressed by light cells (Tabata *et al.*, 1995; Menco *et al.*, 1997).

There may be a difference in the ratio of dark to light cells in the Sprague–Dawley (SD) rat (present experiment) in comparison with the Holtzman strain examined in earlier studies (Farbman, 1980). Cell counts in the vallate papilla of

SD rats showed a dark cell/light cell ratio of 1.3:1 (Pumplin *et al.*, 1997), whereas counts on Holtzman rats showed ratios ranging from 1.6:1 to 3.5:1 (Farbman, 1980). If such a strain difference exists, then the failure of the BrdU-labeled cells to decline below 50% of their peak value by 10.5 days (Table 1) could be attributable to a larger proportion of longer-lived light cells in this strain of rat.

Estimates of the life span of taste cells in rodent fungiform (Beidler and Smallman, 1965) and vallate papillae (Conger and Wells, 1969; Farbman, 1980) put that number at 9–10.5 days. In a specific examination of taste cell types in the rat, Farbman (1980) concluded that dark cells have a life span of ~9 days whereas light cells have a longer but, in his experiment, unspecified life span. BrdU-labeled cells in the present experiment that were not double-labeled for α-gustducin showed a significant decline from their peak number by day 8.5, whereas those that were also labeled for α-gustducin did not significantly decline over the 4 days following their maximum (Table 1). The majority of cells that were not labeled for α-gustducin were probably dark (Type I) cells, since that cell type comprises a majority of the cells in rat vallate taste buds (Farbman, 1980; Pumplin *et al.*, 1997), and the α-gustducin-expressing cells are light cells (Tabata *et al.*, 1995; Boughter *et al.*, 1997; Menco *et al.*, 1997). Although these BrdU-labeled cells without α-gustducin expression do not fall to 50% of their peak value by day 10.5, it is possible that the dark cells within this group (but perhaps not the light cells) have a life span similar to that calculated from [^{3}H]thymidine-labeling experiments. On the other hand, the cells expressing α-gustducin may be longer lived, as they show considerably less decline from their peak value by 10.5 days after BrdU injection.

Estimates of the life span of taste bud cells in this experiment and others (Beidler and Smallman, 1965; Conger and Wells, 1969; Farbman, 1980; Delay *et al.*, 1986) assume that cells dividing at the time of BrdU injection are labeled and that cell counts over the several days following BrdU incorporation represent the first-generation daughter cells of those proliferating stem cells. There is, however, the possibility that some of the cells counted in these experiments could have been second- or third-generation daughter cells. The study by Farbman (1980) followed tritiated thymidine-labeled cells for >25 days and his data showed that the number of labeled cells reached peaks at 6.5, 13.5 and 20.5 days. These successive peaks, which contained fewer cells with each cycle, were interpreted as resulting from second- and third-generation cells entering the taste bud and peaking at 13.5 and 20.5 days. Given those data, it is not likely that any of the cells counted in the present experiment represent second- or third-generation daughter cells, although we cannot rule out such a possibility.

Earlier investigators also suggested that some cells within the taste bud have a much shorter and others a much longer life span (Beidler and Smallman, 1965). The apparent differences in the life span of Type I and Type II cells in the rat vallate papilla have been taken as evidence that these cells comprise two distinct cell lineages within the taste bud (Farbman, 1980). This conclusion is strengthened by a recent morphometric analysis that demonstrated two distinct cell types in the rat vallate taste buds based on multivariate analyses of several morphological indices, including cell size and shape, and the density, shape and degree of invagination of the nuclei (Pumplin *et al.*, 1997). In addition, mosaic analysis of mouse tongue epithelium suggests that taste buds are polyclonal structures, wherein there is more than one cell lineage (Stone *et al.*, 1995). On the other hand, [^{3}H]thymidine labeling on HVEM sections through the mouse vallate papilla suggested that cells differentiate from basal cells to dark, through intermediate, to light cells (Delay *et al.*, 1986), indicating a single cell lineage. The present data on the rat vallate papilla show that α-gustducin expression, which is limited to light (Type II) cells, appears as early as 2.5 days, at which point there were no ^{3}H-labeled light cells in the mouse. Light cells are beginning to express α-gustducin at a time when dark cells are still increasing rapidly in number (see Table 1 and Farbman, 1980). Farbman's data showed light cell incorporation of [^{3}H]thymidine as early as 1.5 days, but certainly by 2.5 days in rat vallate taste buds. These discrepancies may reflect species differences, although it should be noted that data from the present experiment and the earlier data on the rat were based on much larger samples than those on the mouse (Delay *et al.*, 1986).

Both behavioral and electrophysiological studies with α-gustducin knockout mice strongly implicate this G-protein subunit in the transduction of both bitter- and sweet-tasting substances (Wong *et al.*, 1996), although its mechanisms of action in these separate events are not yet understood (Kinnamon, 1996; Lindemann, 1996). Data in the present experiment confirm that α-gustducin is expressed only by cells with morphological characteristics of light cells and further suggest that these cells are longer lived than other cell types, particularly dark cells, which have been shown to have a life span of ~9 days (Farbman, 1980). Therefore, receptor cells with G-protein-coupled second messenger transduction cascades may have a longer life span than other cells in the taste bud.

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