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Exogenous EGF and BDNF treatment enhances differentiation and proliferation of cultured taste cells

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Aim: To determine the effects of epidermal growth factor (EGF) and brain-derived neurotrophic factor (BDNF) on taste cell generation by utilizing a long-term rat taste cell culture that exhibits molecular and functional characteristics of mature taste cells. The role of neurotrophic factors in taste cell development is not well understood.

Materials and methods: A long-term rat taste cell culture exhibiting molecular and functional characteristics of mature taste cells was used. The effects of 3 different concentrations of EGF and BDNF on taste cell differentiation and proliferation were examined at 3 different time points.

Results: We showed the presence of BDNF, EGF receptor, and Trk-B immunoreactivity in subsets of cultured taste cells in vitro. EGF at 10 ng/mL increased the proliferation rate of cultured rat taste cells in 1- and 2-week-old cells. No treatment altered the proliferation rate at 4 weeks. Staining with bromodeoxyuridine (BrdU) and gustducin antibodies demonstrated differentiation in a subset of taste cells, indicating that a 10-ng concentration each of EGF and BDNF increased BrdU and gustducin immunoreactive cell numbers at 1 and 2 weeks.

Conclusion: Our data suggest that EGF and BDNF induced not only taste cell proliferation but also differentiation without requiring nerve innervation.

Key words: EGF, BDNF, taste cell, culture, proliferation, differentiation

Introduction

Taste receptor cells are a highly specialized, heterogeneous population with unique histological, molecular, and physiological characteristics. While much is known about the biological and behavioral basis of taste, comparatively less is understood about the factors governing proliferation and differentiation of taste receptor cells. There are presently 2 models to explain the development of taste cells. The neural induction model postulates that peripheral nerve fibers induce taste bud formation (1,2). Consistent with this, studies of lingual explants and tongue cultures show that denervated mammalian tongues failed to form taste buds, indicating that the structural integrity of the mammalian taste bud is apparently dependent upon the presence of gustatory nerves (cranial nerves VII, IX, and X) (1-5). In another study, damaging cranial nerve IX (the glossopharyngeal nerve) resulted in reduced expression of taste cell markers such as T1R3, gustducin, Mash1, shh, and Nkx2.2 (6-8). Alternatively, according to the early specification model, taste cells may arise from multiple progenitors of the local ecto- or endodermal epithelium of the oropharynx in the absence of innervation (9-12). The role of factors governing proliferation and differentiation of

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taste receptor cells is less understood. Neurotrophins may serve as local trophic factors for the embryonic growth of nerve fibers to induce differentiation of the taste buds (13,14). Brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) are among a number of trophic factors implicated in taste bud development, and BDNF and epithelial growth factor receptor (EGFR) expression were seen in taste buds and gustatory papillae in humans (15-17). In a study of embryonic tongue culture, treatment with EGF resulted in epithelial cell proliferation between papillae, but it decreased papillae formation by inducing activation of EGFR followed by induced PI3K/Akt, MEK/ERK, and p38 MAPK signaling pathways (18). BDNF mRNA is expressed in early developing taste papillae and is essential during embryonic initiation and the maintenance of gustatory innervation of taste buds (19-22). Recently it was shown that BDNF produced in peripheral target cells regulates the survival of early geniculate neurons by inhibiting the cell death of differentiated neurons on E13.5 of development (22). In another study, BDNF (-/-) knockout mice showed a significant reduction in geniculate neurons innervating the tongue, clearly indicating the role of BDNF (23). EGF also appears essential for the normal development of the anterior gustatory epithelia, as both EGF/BDNF knockout and EGF knockout mice exhibit impaired fungiform taste papillae (24). EGF has also been implicated in the proliferation of oral epithelia and appears to play a role in normal taste bud maintenance (25). This role is supported by the observation that EGF was sufficient to reverse the loss of normal fungiform papillae morphology and taste buds following the removal of submandibular and sublingual salivary glands (26).

Results from our initial study generated an in vitro culture system that can be used to maintain rat primary taste cells for more than 2 months (27). In the current study, we utilized this model system to examine the role of exogenous BDNF and EGF on the proliferation/differentiation and physiological properties of cultured rat taste cells. Our results indicate that while exogenous EGF (10 ng/mL) induced proliferation and differentiation, BDNF (10 ng/mL) reduced proliferation in a dose-related manner but increased molecular and functional indicators of differentiation of cultured rat taste cells at 1 and 2 weeks.

Materials and methods

Animal subjects

Sprague Dawley rats, aged 4 to 8 weeks, were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed individually under 12-h light/dark cycles. The rats were given food and water ad libitum. The numbers of animals used were the minimum required to accomplish the goals of this study. All treatment and surgical procedures were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The use and handling of animals was approved by the Monell Chemical Senses Center Institutional Animal Care and Use Committee and was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Primary cell culture

A long-term rat taste cell culture was established according to previously described protocols (27). Briefly, dissected rat tongue was placed immediately into an isolation solution for 5-10 min on ice. The preparation was then removed from ice, and approximately 1 mL of the isolation buffer mixed with 1.5 mg/mL pronase E (Sigma, St. Louis, MO, USA) and 1 mg/mL elastase (Sigma) was injected uniformly with a 25-gauge NORM-JECT syringe (Restek Corporation, Bellefonte, PA, USA) under and around the lingual epithelium of the circumvallate and foliate papillae of the dissected tongue. After 30 min of incubation in the isolation buffer at room temperature, the epithelium was gently peeled from the underlying muscle layer under a dissecting microscope (Stereomaster, Fischer Scientific, Pittsburgh, PA, USA) and placed in more isolation solution. The isolated epithelium was transferred to Iscove's modified Dulbecco's medium (Gibco BRL, New York, NY, USA) containing 10% fetal bovine serum (FBS; BTI, Stoughton, MA, USA) and MCDB 153 medium (Sigma) at a ratio of 1:5. It was then cut into small pieces with a razor blade. The pieces were seeded onto glass coverslips (Fisher Scientific) coated with rat tail collagen type 1 (diluted 1:4 in distilled nuclease-free water; BD Sciences, San Diego, CA, USA) and incubated at 36 °C in a humidified environment containing 5% CO_2. Culture medium was replaced after 48 h and then every 5-7 days. Prior

to use, coverslips were treated with 2 M NaOH for 1 h and left overnight in 70% nitric acid (HNO_3). After 1 h in HCl acid wash, the coverslips were autoclaved in water, rinsed with 70% ethanol and 100% ethanol, and air dried.

Treatment of cultured taste cells with EGF and BDNF

Taste cells were cultured and maintained as described above. Rat taste cells were cultured for 1, 2, and 4 weeks, and then EGF (01-101, Upstate Biotechnology, Lake Placid, NY, USA) and BDNF (01-194, Upstate Biotechnology) were added, either individually or in combination, exogenously into tissue culture medium for 5 days at 3 different concentrations (1, 10, and 100 ng/mL). During this period, if cells were used for the determination of gustducin expression, taste cells were also labeled with BrdU as described below. Cultured taste cells were tested at 5 days after plating. Immunostaining of EGF- and BDNF-treated cultured cells were analyzed as described below.

Determination of cellular proliferation: WST-1 proliferation assay

Cellular proliferation was assessed using the reagent WST-1 cell proliferation kit (Chemicon, Billerica, MA, USA). This colorimetric assay measures the metabolic activity of viable cells based on the cleavage of the tetrazolium salt WST-1 into formazan by mitochondrial dehydrogenase in live cells (28). Primary taste cells were plated in a 96-well plate and maintained until the time indicated for exogenous treatment. Cells were treated with EGF, BDNF, or a combination of the 2 (EGF+BDNF) at the indicated concentrations (0, 1, and 10 ng/mL) for 5 days. This was followed by incubation with WST-1 reagent at a dilution of 1:10 in the original conditioned media for 4 h. After thorough shaking for 1 min, the formazan produced by the metabolically active cells in each sample was measured at a wavelength of 450 nm with a plate reader (ICN Multiscan MCC/340, MKI; ICN Biomedicals GmbH, Meckenheim, Germany). Absorbance readings were normalized against control wells containing medium only. In order to optimize the differences in each well and to compare each of the conditions, total protein concentration was estimated for each well using the Bio-Rad DC Protein Estimation Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The statistical significance of the doubling rate between the groups was determined by one-way ANOVA and Tukey's test using the StatMost™ software program (Dataxiom Software Inc., Los Angeles, CA, USA).

Immunohistochemistry of rat tongue foliate papilla

Rats were euthanized by CO_2 inhalation followed by cervical dislocation. The tongue was dissected proximal to the circumvallate papillae and close to the foliate papillae region, then immediately placed into 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.2) for 1 h at room temperature. After washing 3 times with PBS, tissue was placed in 10%, 20%, and 30% sucrose solutions, each for 24 h at 4 °C for cryoprotection. The tissues were rapidly frozen in M-1 embedding matrix (Shandon Lipshaw, Pittsburgh, PA, USA) in a dry-ice acetone bath. Sections were cut horizontally at a thickness of 8 μm through the entire foliate papillae and serially mounted onto SuperFrost Plus slides (Fisher Scientific) with 4 sections per slide. Slides were stored in slide boxes at either −30 or −80 °C prior to use. After being maintained at 40 °C for 1 h, tissues were initially treated with H_2O_2 solution (4 mL PBS + 0.5 mL 100% methanol + 0.5 mL 30% H_2O_2) for 20 min to block endogenous peroxidase. Tissues were then blocked with 0.3% Triton X-100, 4% normal goat serum, and 3% bovine serum albumin in PBS for 1 h. The primary antibodies, polyclonal rabbit anti-Trk-B (1:250, sc-12; Santa Cruz Biotechnology, Santa Cruz, CA, USA; raised against a peptide corresponding to the C-terminal cytoplasmic domain of mouse Trk-B) or polyclonal rabbit anti-EGFR (1:250, sc-03; Santa Cruz Biotechnology; raised against a peptide corresponding to the C-terminus of EGFR of human origin), were diluted in blocking solution, added to the tissue-covered slides, and incubated overnight at 4 °C in a humidified chamber. After washing 3 times with PBS for 15 min, the tissues were reacted in the dark with goat antirabbit Alexa 633 for Trk-B and EGFR (1:500; Molecular Probes Inc., Eugene, OR, USA) and diluted in blocking buffer for 30 min at room temperature in a humidified chamber. The slides received 3 PBS washes of 15 min each followed by 3 washes with water of 10 min each prior to mounting with VECTASHIELD with DAPI

(Vector Labs, Burlingame, CA, USA). Controls for immunofluorescence consisted of omitting the primary antibody (only using the secondary antibody) and substituting with nonimmune serum appropriate for the primary antibody.

BrdU labeling and immunocytochemistry

In order to determine the relationship between proliferation/differentiation and expression of Trk-B and EGFR in primary cultured taste cells, we examined the expression of Trk-B and EGFR immunocytochemically in both 9-day-old and 2-month-old cultures. To determine whether proliferating cells expressed these growth factors or receptors, cells were treated with BrdU. For immunofluorescence double-labeling, isolated taste cells were seeded onto collagen-coated coverslips, maintained for the indicated time, and treated with 50 μM BrdU (Sigma) dissolved in DMSO (Sigma) for 48 h. BrdU was then replaced with fresh medium. The BrdU-treated cells were maintained for 3 additional days in culture and were then fixed with 4% paraformaldehyde in PBS (pH 7.2) for 10 min at room temperature. After washing in PBS, the cells were treated with H_2O_2 solution for 20 min to block endogenous peroxidase and then denatured with 2 N HCl at 37 °C for 30 min. SuperBlock blocking buffer in PBS (Pierce Chemical Company, Rockford, IL, USA) was used to reduce nonspecific binding. Cells were then incubated overnight at 4 °C with mouse anti-BrdU (dilution 1:100; Sigma B-2531) diluted in 10% SuperBlock with 0.05% Tween 20. Alexa Fluor 488 conjugated antimouse IgG (1:500; Molecular Probes Inc.) diluted in 10% SuperBlock with 0.05% Tween 20 was used for immunofluorescence detection. Coverslips were thoroughly washed with PBS and then reblocked with 0.3% Triton X-100, 4% normal goat serum, and 3% bovine serum albumin in PBS and incubated with either polyclonal rabbit anti-Trk-B or polyclonal rabbit anti-EGFR (1:250) overnight at 4 °C. After washing with PBS, cells were incubated with Alexa Fluor 633 goat antirabbit-IgG (1:500; Molecular Probes Inc.) diluted in blocking buffer for 1 h. After washing in PBS and water, coverslips were mounted with VECTASHIELD (Vector Laboratories). To determine the specificity of staining, controls for immunofluorescence consisted of omitting the primary antibody or substituting the primary antibody with the host IgG from which the antibody was generated. Controls for double-labeling experiments involved omitting either the first or second primary antibody while maintaining all other steps in the protocol to check for nonspecific interactions. In all cases, these controls revealed no artifactual labeling. Immunoreactive cells were counted in at least 3 fields at 20× magnification.

Confocal imaging

Fluorescent images were captured with a Leica TCS SP2 spectral confocal microscope (Leica Microsystems Inc., Mannheim, Germany) using UV, argon, and HeNe lasers and a HC PL APO CS 20.0× (0.070 NA) objective. The excitation wavelengths used were at 405 nm for DAPI, 488 nm for Alexa Fluor 488, and 633 nm for Alexa Fluor 633, with emissions detected at appropriate wavelengths. The pinhole diameter was set at the first minimum diameter of the Airy disk for the objective used, which provided acceptable resolution of the z-axis for the fluorescent focal plane. Laser power and photomultiplier gain were adjusted for an optimal signal-to-noise ratio and held constant for comparison of antibody-labeled and control slides. Sequential acquisition of each wavelength was used for some double-labeling experiments to prevent crosstalk or bleed-through between fluorophores. Leica Scanware software was used to acquire confocal images, scanning unidirectionally with a 1024 × 1024 pixel format with 3 lines and 2 frames on average. Computer-controlled digital zoom was used to increase magnification to a maximum of 2.3× with the 20× objective. Images were arranged and minimally adjusted for contrast and brightness using LCS software (Leica) and Adobe Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA, USA).

Western blot

Western blots were conducted using standard immunoblotting techniques (29). Cultured primary rat taste cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) containing protease inhibitors. Protein concentration was estimated for each sample using the Bio-Rad DC Protein Estimation Kit (Bio-Rad

Laboratories) according to the manufacturer's protocol. Protein samples were mixed with SDS loading buffer containing β-mercaptoethanol; the mixture was boiled for 5 min and then placed on ice for 5 min. The cellular lysates were separated by SDS-polyacrylamide (5%-15%) gradient gel (Bio-Rad Laboratories) electrophoresis and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories) that was then incubated at 4 °C overnight with 1% nonfat dry milk. Polyclonal rabbit anti-Trk-B (1:250; Santa Cruz Biotechnology) and polyclonal rabbit anti-EGFR (1:250; Santa Cruz Biotechnology) were used to identify taste cell proteins. After 1.5 h of incubation with primary antibodies at room temperature, the membrane received 3 washes of 15 min each in PBS with 0.05% Tween-20 (PBS/T). It was then reacted with horseradish peroxidase (HRP)-conjugated secondary antirabbit antibody (NA934, 1:5000; Amersham Biosciences, Piscataway, NJ, USA) for 1 h at room temperature, followed by 3 washes of 15 min each with PBS/T. Signals were detected with the enhanced chemiluminescence (ECL) immunoblot detection system (Amersham Biosciences) following the manufacturer's instructions. X-ray films were later scanned for documentation and analysis.

Statistics

Results are expressed as means ± SE calculated from 3 independent experiments per group. Comparisons between control and treatment groups were performed by 2-way ANOVA (StatMost™ software program, Dataxiom Software Inc.) followed by Tukey's test. $P < 0.05$ was considered significant.

Results

Expression of Trk-B and EGFR in rat foliate papillae and cultured rat taste cells

To explore the effects of neurotrophic factors that may be involved in taste cell generation, we examined the expression of several molecules previously implicated in this process (24,30-32). Immunohistochemical and immunocytochemical experiments were performed to determine the presence of EGFR and Trk-B in rat foliate papillae tissue and cultured rat taste cells aged 9 days or 2 months. Immunoreactivity for EGFR or Trk-B was detected in cells within the foliate papilla of rat tongue (Figures 1A and 1B, respectively). The Trk-B antibodies immunolabeled subsets of cells within the cultures at 9 days and after 2 months (Figures 1C and 1D, and 1E and 1F, respectively). We also observed EGFR immunoreactivity in the cultured taste cells at 9 days and after 2 months (Figures 1G and 1H, and 1I and 1J, respectively). The staining patterns observed in rat tongue tissue were consistent with those reported in the literature (24,26,31,33,34).

We utilized cultured rat foliate and circumvallate taste cells cultured for 9 days to determine whether cells expressed EGFR and Trk-B. Western blot analysis of cultured rat taste cells obtained from rat circumvallate and foliate papillae identified bands of the appropriate size, indicating expression of EGFR and Trk-B, albeit at apparently different levels of expression (these were not quantified) (Figure 1K).

Expression of Trk-B and EGFR proteins in a subset of newly divided cultured taste cells

Immunocytochemical experiments were performed to determine whether cells expressing Trk-B or EGF-R were actively dividing or differentiated. Taste cell proliferation was demonstrated by labeling with BrdU. Immunocytochemical experiments showed that a subset of BrdU-labeled cells also exhibited EGFR or Trk-B immunoreactivity in the 9-day-old and 2-month-old cultures (Figure 2). Approximately 30% of cells were labeled with BrdU after 9 days in culture, indicating cellular proliferation (Table 1). A subset of these cells were immunoreactive for both Trk-B and BrdU (approximately 20%-25% of all cells), while approximately 10% and 20% of cells were labeled selectively with either BrdU or Trk-B (Figures 2A-2D). After 2 months, the presence of BrdU-immunoreactive cells decreased to 10% of the total cells, suggesting a decline in proliferation with time (Figures 2I-2L). A subset of 2-month-old cells was successfully labeled with either Trk-B (7%-10%) or BrdU + Trk-B antibodies (5%-7%), indicating that at least some newly generated cells could be responsive to BDNF.

We observed similar results using EGFR antibody in cells labeled with BrdU at 9 days and 2 months of age. BrdU-treated 9-day-old taste cells showed

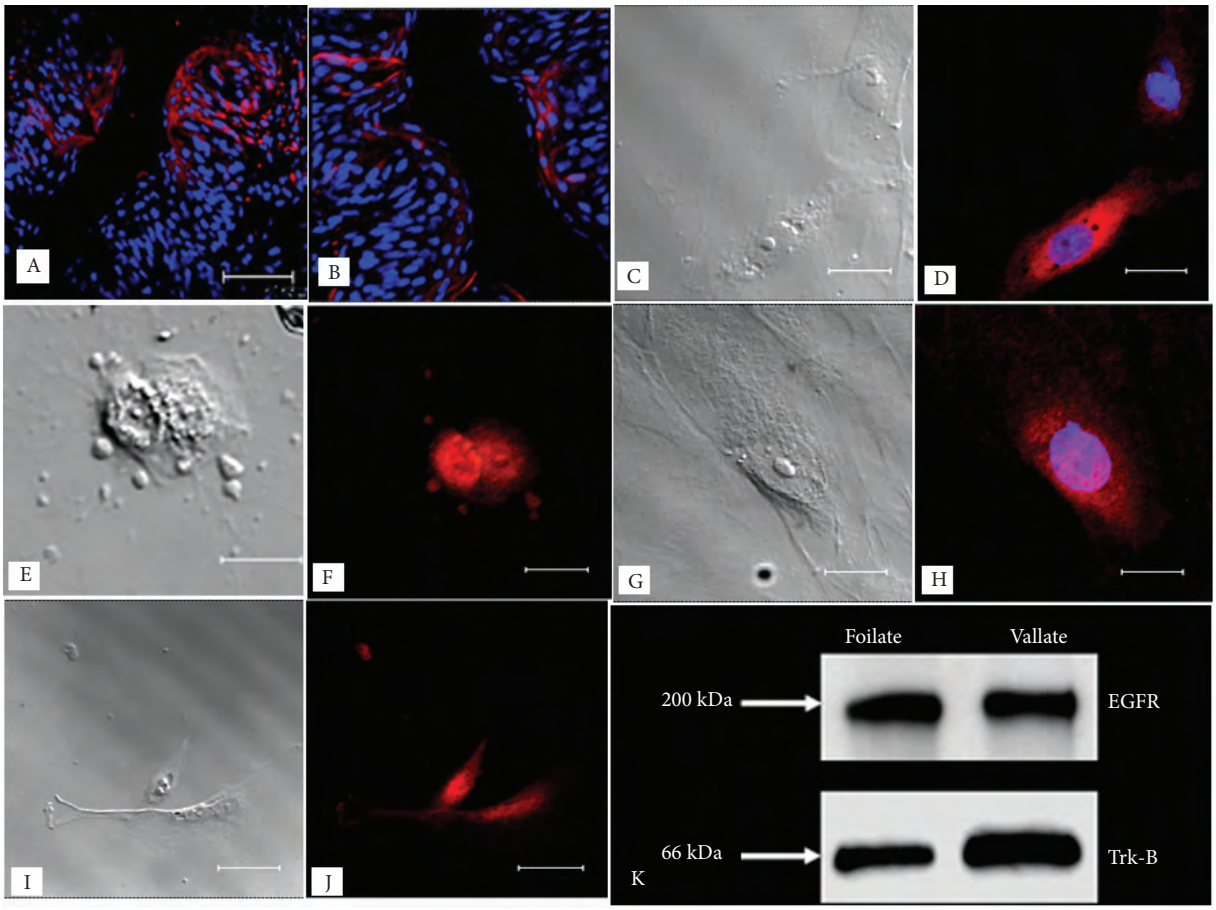

Figure 1. EGFR and Trk-B immunoreactivity in taste papillae and taste cells in culture: A) EGFR and B) Trk-B immunoreactivity in rat taste bud cells from foliate papillae; C) bright-field and D) immunofluorescence images of cultured taste cells immunostained for Trk-B at 9 days; E) bright-field and F) immunofluorescence images of cultured taste cells immunostained for Trk-B at 2 months; G) bright-field and H) immunofluorescence images of cultured taste cells immunostained for EGFR at 9 days; ; I) bright-field and J) immunofluorescence images of cultured taste cells immunostained for EGFR at 2 months; nuclei were stained with DAPI (blue); scale bar = 30 μm. K) Western blot analysis indicating the presence of Trk-B and EGFR expression at the expected size in cultured taste cells.

immunoreactivity to both EGFR and BrdU (20%) (Figures 2E-2H). Approximately 15% of cells in these cultures were immunoreactive only to EGFR, while less than 1% of cells were labeled only with BrdU, indicating that almost all newly generated cells were EGFR positive. BrdU labeling was lower in 2-month-old cultured taste cells, approximately 10% of the total cells, suggesting a decline in proliferation with time. A subset of 2-month-old cells showed immunoreactivity with either EGFR alone (<1%) or BrdU alone (4%-5%). Double-labeling of BrdU and EGFR antibodies produced 5% positive cells, indicating that about 50% of the newly generated cells could be responsive to EGF (Figures 2M-2P).

These results showed that double-labeling of either Trk-B or EGFR with BrdU provides approximately 25% positive staining at 9 days and 5% at 2 months. Some cultured cells were immunoreactive to a single biomarker only, and many cells were not immunolabeled with any of these antibodies. The proportion of double-labeled cells relative to total BrdU-labeled cells was comparable at both time points. This indicates that a quantitative decline in growth occurs over this time frame in culture, although some cells with the potential to respond to neurotrophic factors persist.

The specificity of immunostaining was evaluated in a variety of control experiments. No cross-reactivity

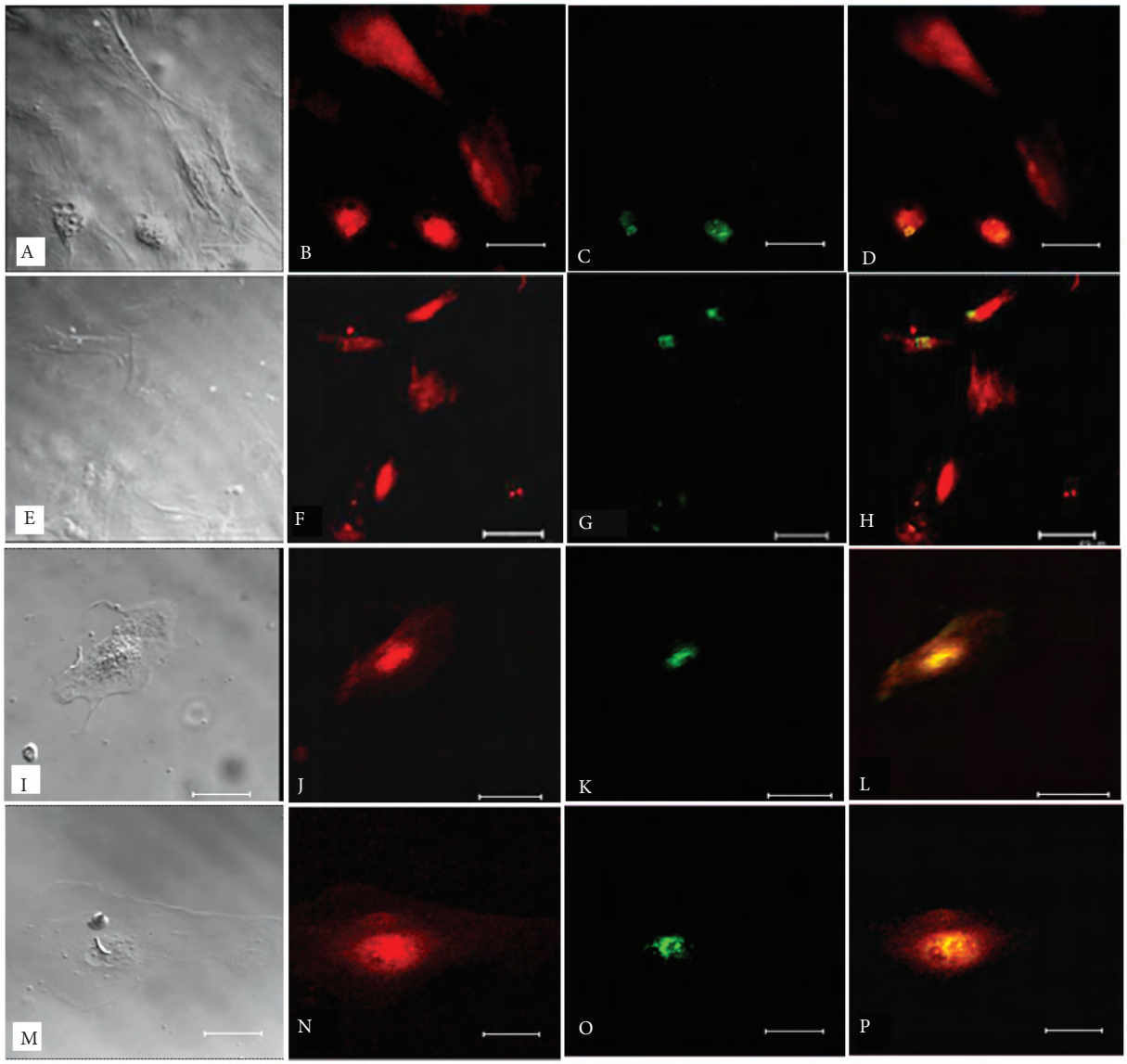

Figure 2. Immunocytochemical experiments demonstrated that a subset of BrdU immunoreactive cells also exhibited EGFR or Trk-B immunoreactivity in the 9-day-old and 2-month-old cultures. A subset of cells treated with BrdU was immunoreactive for both Trk-B (red) and BrdU (green) in both 9-day-old (Figures 2A-2D) and 2-month-old (Figures 2I-2L) cultures. Similarly, EGFR immunostained a subset of BrdU-labeled cells in both younger and older cultures. BrdU-treated 9-day-old and 2-month-old taste cells showed immunoreactivity to both EGFR (Figures 2E-2H) and BrdU (Figures 2M-2P). Scale bar = 30 μm.

Table 1. Counts of in vitro-generated taste cells expressing receptors for EGF and BDNF.

Nine-day-old cells (n = 200)			**Two-month-old cells (n = 140)**		
EGFR +	BrdU +	20%	EGFR +	BrdU +	5%
EGFR –	BrdU +	<1%	EGFR –	BrdU +	4%-5%
EGFR +	BrdU –	10%-20%	EGFR +	BrdU –	<1%
Trk-B +	BrdU +	20%-25%	Trk-B +	BrdU +	5%-7%
Trk-B -	BrdU +	5%-10%	Trk-B –	BrdU +	3%
Trk-B +	BrdU –	20%	Trk-B +	BrdU –	7%-10%

with the secondary antibody (Alexa 633) was detected when the BrdU-reacted cells were further processed in the absence of the second primary antibody. Cells not exposed to BrdU showed no BrdU immunoreactivity, demonstrating the specificity of the BrdU antibody (data not shown). Immunostaining with antibody specific immunoglobulin did not indicate nonspecific immunoreactivity (data not shown).

Determination of taste cell proliferation after treatment with EGF and BDNF

Effects of EGF and BDNF on cellular proliferation of 1-week-old cultured taste cells

In 1-week-old cultured taste cells (Figure 3A), EGF at 10 ng/mL showed significant effects on cellular proliferation compared with the control (0 ng/mL), 1 ng/mL, and 100 ng/mL treatments ($P = 2 \times 10^{-6}$,

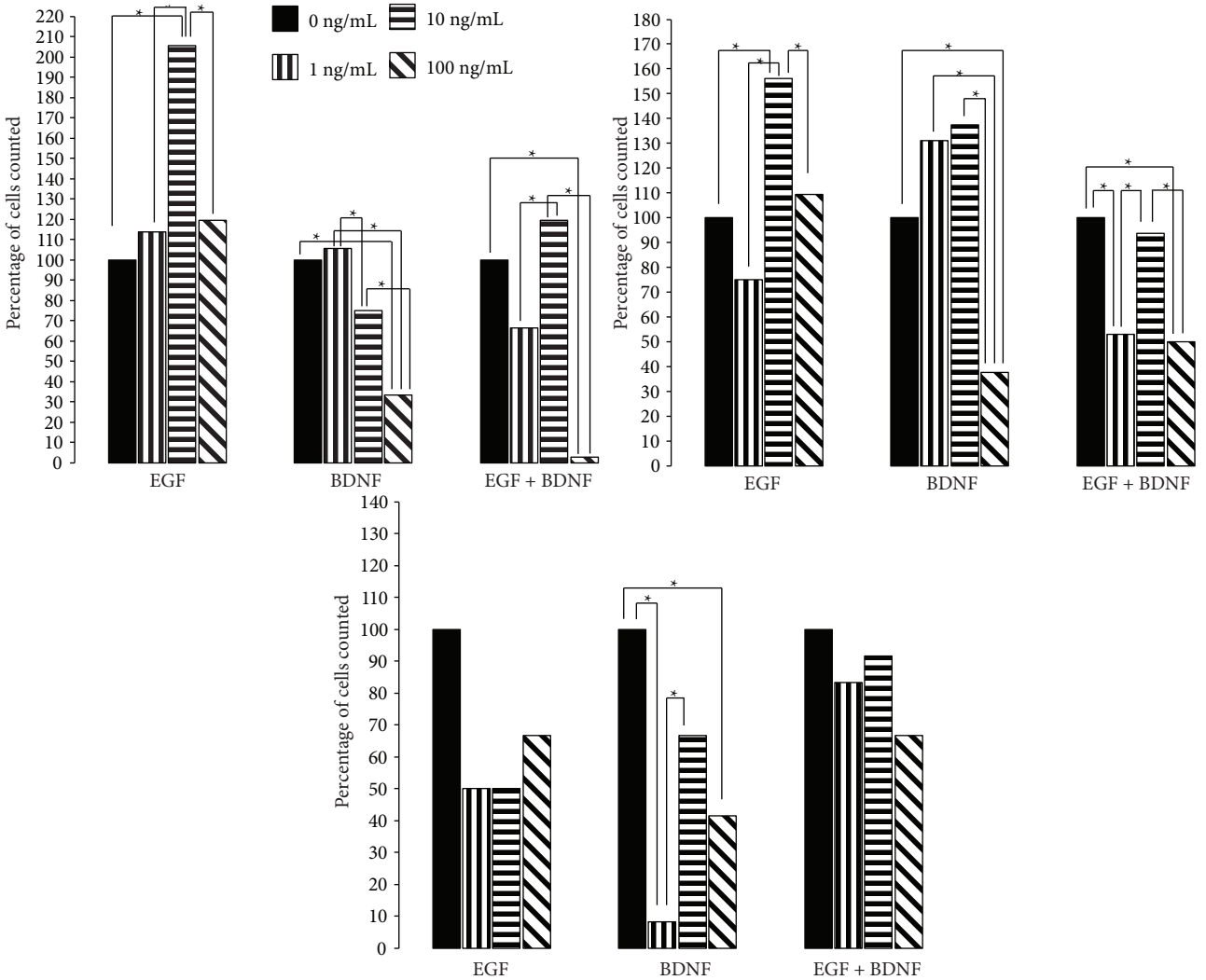

Figure 3. EGF and BDNF modulation of taste cell proliferation in culture is dose- and age-dependent. In 1-week-old cultures (Figure 3A), EGF at 10 ng/mL showed significant stimulatory effects on cellular proliferation compared with other groups. BDNF treatment dose-dependently inhibited cellular proliferation. The addition of EGF did not attenuate the antiproliferative effect of BDNF. In 2-week-old cultures (Figure 3B), EGF-treated cells showed patterns similar to those of the 1-week-old cells. BDNF at 100 ng/mL significantly inhibited cellular proliferation compared with the other groups. Treatment with EGF+BDNF did not show any proliferative effect at any doses compared to the control; however, treatment with both factors at 1 and 100 ng/mL resulted in reduced cellular proliferation. In 4-week-old cultures (Figure 3C), there were no significant effects of EGF alone or in combination with BDNF on proliferation compared with the control. As in younger cells, treatment with BDNF alone at 1 and 100 ng/mL significantly suppressed proliferation of cultured taste cells compared with untreated cells.

dfn = 3, dfd = 12). BDNF treatment exhibited an antiproliferative effect, which was significant at 100 ng/mL compared to other groups ($P = 7 \times 10^{-7}$). Although there was no difference between the control and treatment groups at 1 ng/mL and 10 ng/mL, there was a significant difference between the treatment groups ($P = 7 \times 10^{-7}$). Combined treatment with EGF+BDNF at 100 ng/mL reduced proliferation relative to the control or 10 ng/mL, but not 1 ng/mL, suggesting a reverse U-shaped dose relationship ($P = 4 \times 10^{-5}$).

Effects of EGF and BDNF on proliferation of 2-week-old cultured taste cells

At 2 weeks, EGF-treated cells showed patterns similar to those of the 1-week-old cells. A significant proliferative effect was elicited by 10 ng/mL EGF, while there was no significant difference between 1 ng/mL and 100 ng/mL and the control ($P = 4 \times 10^{-5}$, dfn = 3, dfd = 12). In BDNF-treated cells, 1 ng/mL and 10 ng/mL induced cellular proliferation slightly, but not significantly, compared with the untreated cells. Proliferation was significantly reduced by treatment with BDNF at 100 ng/mL compared to treatment with 1 ng/mL, 10 ng/mL, and the control ($P = 8 \times 10^{-6}$). In contrast to results for 1-week-old cells, treatment with EGF+BDNF did not promote proliferation at any dose compared to the control; however, cells treated with 1 ng/mL and 100 ng/mL exhibited significantly less proliferation than untreated cells ($P = 0.0003$).

Effects of EGF and BDNF on cellular proliferation of 4-week-old cultured taste cells

There were no significant effects of EGF treatment on cellular proliferation compared with untreated control cells cultured for 4 weeks. Treatment with BDNF at 1 ng/mL and 100 ng/mL significantly suppressed proliferation of cultured taste cells compared with untreated cells ($P = 3 \times 10^{-6}$, dfn = 3, dfd = 12), while treatment with 10 ng/mL BDNF did not affect proliferation. Similarly, treatment with EGF+BDNF did not alter proliferation compared with untreated controls.

At 1 and 2 weeks of culture, cell proliferation was modulated by growth factor treatments (Figures 3A and 3B). While 10 ng/mL EGF induced proliferation, BDNF treatment at 1 ng/mL or 100 ng/mL inhibited proliferation. When combined, inhibition was observed at 1 ng/mL and 100 ng/mL. In contrast, proliferation in 4-week-old cultures was insensitive to these growth factor treatments (Figure 3C).

Effects of EGF and BDNF on cultured taste cell differentiation

Cultured taste cells treated with BrdU in the presence of neurotrophic factors EGF, BDNF, or both showed contrasting dose- and time-related effects on the differentiation of cultured taste cells. Cells were seeded onto collagen-coated coverslips and maintained for 1, 2, or 4 weeks. Cells were treated with 50 mM BrdU in the presence of EGF, BDNF, and EGF+BDNF at 3 different concentrations (1, 10, and 100 ng/mL) for 5 days and fixed with 4% paraformaldehyde. To determine the impact of these treatments on differentiation and proliferation, cells were stained with anti-gustducin and anti-BrdU antibodies, respectively, and visualized by confocal microscopy. The quantity of cells counted for each condition (control versus treated) was similar, but the number varied across groups (1 week versus 2 weeks versus 4 weeks). Therefore, in order to standardize cell differentiation at the indicated time points, the values were expressed as a percentage of cells counted. Untreated cells (1 week) exhibited a profile of proliferation and differentiation as follows: 23% BrdU − and gustducin −, 40% BrdU − and gustducin +, 9% BrdU + and gustducin −, and 28% BrdU + and gustducin + (Table 2A). No treatment produced a higher proportion of gustducin + or BrdU + cells when treated at 1 week. Treatment with 1 ng/mL of EGF did not show any effect on these parameters, but BDNF treatment resulted in a higher prevalence of BrdU − and gustducin + cells, with an increase from 40% to 51%. In contrast, treatment with 1 ng/mL EGF+BDNF resulted in an increase in BrdU − and gustducin − cells from 23% to 41% and a decrease in BrdU + and gustducin + cells from 28% to 9%. Treatment with 10 ng/mL EGF, BDNF, or EGF+BDNF resulted in a moderate increase in BrdU − and gustducin + cells from 40% in the control to 60%, 65%, and 65%, respectively. Treatment with EGF, BDNF, and EGF+BDNF at 10 ng/mL reduced the proportion of BrdU − and gustducin − cells significantly (Table 2A). EGF did not alter the rate of

Table 2. Effects of exogenous EGF and BDNF on taste cell differentiation and proliferation.

Growth factor	Dose ng/mL	Age (weeks)	Total cells (n)	% both negative	% BrdU +, gustducin –	% BrdU –, gustducin +	% both positive
A (1 week)							
Control	0	1	128	23	9	40	28
EGF	1	1	79	24	1	48	27
EGF	10	1	146	5	2	60	33
EGF	100	1	200	60	2	18	21
BDNF	1	1	129	13	3	51	33
BDNF	10	1	154	1	2	65	32
BDNF	100	1	114	29	11	44	17
EGF+BDNF	1	1	151	41	1	50	9
EGF+BDNF	10	1	206	1	2	65	32
EGF+BDNF	100	1	208	15	4	66	14
B (2 weeks)							
Control	0	2	32	31	12	44	13
EGF	1	2	15	67	7	13	13
EGF	10	2	61	2	3	61	34
EGF	100	2	38	11	5	58	26
BDNF	1	2	33	30	9	27	33
BDNF	10	2	44	2	0	66	32
BDNF	100	2	59	27	4	39	30
EGF+BDNF	1	2	19	47	5	26	21
EGF+BDNF	10	2	57	4	2	53	42
EGF+BDNF	100	2	64	17	2	40	48
C (4 weeks)							
Control	0	4	77	18	8	42	32
EGF	1	4	76	29	1	54	16
EGF	10	4	81	37	1	40	22
EGF	100	4	NA	NA	NA	NA	NA
BDNF	1	4	27	81	11	4	4
BDNF	10	4	22	14	14	42	30
BDNF	100	4	NA	NA	NA	NA	NA
EGF+BDNF	1	4	24	25	0	63	13
EGF+BDNF	10	4	57	26	9	55	11
EGF+BDNF	100	4	NA	NA	NA	NA	NA

BrdU + and gustducin + taste cells. EGF treatment at 100 ng/mL increased the prevalence of marker negative cells from 23% to 60% and reduced that of BrdU – and gustducin + cells from 40% to 18%, with no change in the prevalence of cells labeled with both markers or with BrdU alone. EGF at 100 ng/mL may have been responsible for an increase in cellular proliferation but may also have reduced the occurrence of BrdU – and gustducin + cells. All treatments, with the exception of 100 ng/mL BDNF, reduced the proportion of BrdU + and gustducin – cells.

At 2 weeks, untreated cells exhibited a profile of proliferating and differentiated cells as follows (Table 2B): 31% BrdU – and gustducin –, 44% BrdU – and gustducin +, 12% BrdU + and gustducin –, and 13% BrdU + and gustducin +. While treatment with 1 ng/mL of EGF and EGF+BDNF significantly increased the proportion of gustducin – cells, treatment with either 10 or 100 ng/mL of EGF shifted the labeling pattern to a smaller number of nonimmunolabeled cells with a higher proportion of gustducin + cells. Cultured taste cells treated with 10 ng/mL of EGF, BDNF, or EGF+BDNF yielded a significant increase in gustducin + cells. Additionally, at 100 ng/mL, EGF, BDNF, and EGF+BDNF increased the prevalence of BrdU + and gustducin + cells (Table 2B).

Untreated cells grown for 4 weeks and treated with BrdU for 5 days exhibited a profile of proliferating and differentiated cells as follows (Table 2C): 18% BrdU – and gustducin –, 42% BrdU – and gustducin +, 8% BrdU + and gustducin –, and 32% BrdU + and gustducin +. Treatment with EGF at 1 ng/mL and EGF+BDNF at 1 and 10 ng/mL significantly increased the proportion of gustducin + and BrdU – cells at 4 weeks. BDNF treatment at 1 ng/mL significantly reduced the prevalence of gustducin + cells and increased the frequency of unlabeled cells. Moreover, EGF at 10 ng/mL induced a higher proportion of BrdU – and gustducin – cells, while EGF showed the opposite effect. The proportion of BrdU + and gustducin – cells was higher when treated with BDNF at either 1 or 10 ng/mL (Table 2C). These data indicate that neurotrophic factors EGF and BDNF influence the proliferation and differentiation of cultured taste cells in a complex but apparently interactive fashion, and that both concentration and cell age modulate these effects.

Discussion

In contrast to the neuronal induction model (1,2), the early specification model suggests that taste cells can arise from the local ecto- or endodermal epithelium of the oropharynx in the absence of innervation, suggesting that taste cells may arise from multiple progenitors (9-12,35,36). Neurotrophins might also act as local trophic factors, inducing differentiation of the taste buds by stimulating embryonic growth of nerve fibers (37,38). However, the relationship between trophic factors and the signaling mechanisms governing differentiation/proliferation and physiological maturation in taste cells is not fully understood. This is due in part to the lack of an in vitro model. In an in vitro model, taste cells lose their innate components such as nerve fibers, surrounding epithelial cells, and saliva. To determine the role of neurotrophic factors on taste cell proliferation and differentiation, we initially showed the presence of EGFR and Trk-B, respectively, in rat foliate papillae and cultured rat taste cells aged 9 days and 2 months, which is consistent with previous in vivo data (17,24,31,39). The expression of BDNF and its receptor Trk-B was found to be necessary for proper morphogenesis of lingual gustatory epithelia, taste bud formation during development, and the regeneration of taste buds after denervation (31,32,40,41). Overexpression of BDNF in mouse tongue epithelium showed that most fungiform taste buds failed to become innervated, and fungiform papillae were small and disappeared postnatally. These results suggest that BDNF, which normally functions as a chemoattractant, has important roles in regulating the development and spatial patterning of fungiform papilla (42). EGFR and Trk-B may have a role in taste cell regeneration. It has been reported that BDNF in taste bud cells may show its effect through Trk-B receptors on the axons of nerves innervating taste buds and may act on surrounding cells (31,43). Ookura et al. reported that EGF stimulated the growth of cultured mouse tongue epithelium (30). EGF is also involved in the regulation of cell proliferation and differentiation in a wide variety of tissues during various stages of embryonic development (44-46). EGF stimulated the proliferation of mouse tongue epithelial cells (30). Our results are consistent with these findings; EGF at 10 ng/mL significantly increased

proliferation of 1- and 2-week-old cultured taste cells but was ineffective in 4-week-old cells. In contrast, BDNF inhibited cellular proliferation in cells at all ages tested, and this inhibitory effect was negated by concurrent treatment with the effective concentration of EGF (10 ng/mL). Similar to EGF alone, EGF+BDNF did not modify the proliferation rates of 4-week-old cells.

To examine differentiation, we used the expression of gustducin as a clear indication of phenotypic similarity to at least one population of mature taste cells in vivo. Additional studies are needed to examine the influence of these neurotrophic factors on cells that may express proteins indicative of other mature taste cell types. Cultured taste cells treated with 50 mM BrdU in the presence of EGF, BDNF, and EGF+BDNF for 5 days indicated that EGF, BDNF, and their combination at 10 ng/mL increased the prevalence of gustducin immunoreactive cells in 1- and 2-week-old taste cell cultures but were ineffective in 4-week-old cultures. Although overexpression of BDNF resulted in disruption of taste bud development and numbers (47,48), EGFR/BDNF double-null mutant mice showed a higher frequency of failed fungiform taste bud differentiation (24). In another study, EGF supplementation reversed the effects of the removal of submandibular and sublingual salivary glands, which resulted in the loss of fungiform taste buds and changes in cellular morphology (26). Interestingly, no treatment produced any significant change in cultured taste cell differentiation and proliferation in 4-week-old cultured rat taste cells. Presumably, in cultures of this age, the number of cells expressing receptors for these neurotrophins is insufficient.

In conclusion, expression of receptors for 2 neurotrophic factors implicated in taste bud development and maintenance in our medium-term culture model facilitates the study of the role of these factors in the molecular processes underlying proliferation and differentiation of taste receptor cells. Here we showed that exogenous EGF and BDNF at certain concentrations affect proliferation and apparent maturation of cultured cells, based on expression of a prominent and specific taste cell marker, gustducin. This work lays the foundation for future studies to examine the mechanisms controlling proliferation and regeneration of cultured taste cells and may provide new insights for future in vivo therapeutic experiments.

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