

Analysis of Neurogenesis in a Mammalian Neuroepithelium: Proliferation and Differentiation of an Olfactory Neuron Precursor In Vitro

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Summary

Development of a culture system for mammalian olfactory epithelium has permitted the process of neurogenesis to be examined in vitro. Antibody markers allowing the unambiguous identification of putative neuroepithelial stem cells (keratin⁺ basal cells) and differentiated neurons (N-CAM⁺ olfactory receptor neurons) are described. In combination with [³H]thymidine uptake analysis, these antibodies have been used to characterize the existence, proliferation, and differentiation of the immediate neuronal precursor in this system. This cell is distinct from basal cells and rapidly sorts out from them, dividing as it migrates. Data are presented which suggest that the precursor follows a simple lineage program, dividing to give rise to two N-CAM⁺ daughter neurons. Although this precursor efficiently generates neurons in defined medium, neurogenesis subsequently ceases because new precursors are not produced, suggesting that epigenetic factors may regulate continual neurogenesis in this system.

Introduction

It is the number of neurons in a nervous system that ultimately determines its complexity. The fundamental process determining neuronal number is the proliferation of neuronal precursor cells and the terminal differentiation of their progeny into postmitotic neurons. In higher vertebrates, the neurons and glia of the nervous system derive from the embryonic neural tube and neurogenic placodes, and for most of the nervous system, it is within these neuroepithelia that neurogenesis takes place (e.g., Sidman and Rakic, 1973; Jacobson, 1978). Studies of [³H]thymidine uptake by neuronal precursors in vivo show that neurogenesis is tightly regulated, both temporally and spatially, during development (e.g., Kauffman, 1968; Mares and Lodin, 1970; Shimada and Langman, 1970; Angevine, 1970). However, because the structures in which neurogenesis takes place exist only transiently and for the most part very early in embryonic development, experimental manipulation of these tissues is difficult. Consequently, very little is known about the cellular regulation or the genetic control of neurogenesis.

To circumvent the problems associated with studying neurogenesis in vivo, we are attempting to reconstitute this process in vitro. The tissue we have chosen to use for these studies is the olfactory epithelium (OE) of the mouse. The OE is a neuroepithelium not unlike the embryonic neural tube in structure. As a tissue for in vitro

studies, however, the OE has significant advantages over the neural tube. First, it is much simpler, giving rise to only a single type of neuron, the olfactory receptor neuron. Second, it retains both its epithelial morphology and the ability to generate neurons throughout adult life (Graziadei and Monti Graziadei, 1978). Third, studies of regenerating olfactory receptor neurons in adult animals have already provided insights into cellular interactions that regulate neurogenesis in the OE (e.g., Graziadei, 1973; Monti Graziadei and Graziadei, 1979; Costanzo and Graziadei, 1983; Camara and Harding, 1984).

This manuscript describes the development of in vitro methods of studying neurogenesis in the mammalian OE and the results obtained so far using this approach. These results suggest that neurogenesis is a multistage process, the final step of which involves a proliferating neuronal precursor not previously identified in this tissue. This precursor can be identified by morphological and immunochemical criteria and by its ability to take up [³H]thymidine, and its behavior in vitro suggests that its adhesion properties differ from those of other cells in the epithelium. Both the precursor and the ability to generate neurons disappear after several days in these cultures, indicating that while the precursor's terminal division and the subsequent neuronal differentiation of its progeny require no factors other than those present in this culture system, continual generation of neurons requires something more. Thus, this in vitro system should prove of value not only for elucidating the cellular stages of neurogenesis, but also for defining epigenetic factors required for the persistence of this process.

Results

Immunocytochemical Studies Establish the In Vitro Counterparts of Basal Cells and Neurons in the Olfactory Epithelium

To study neurogenesis in tissue culture it was necessary to find antibody markers that could distinguish proliferating neural precursors from differentiated neurons.

Studies of neuronal regeneration in the OE in vivo suggested that the basal cells, so named because they lie adjacent to the basement membrane, are self-renewing stem cells that ultimately give rise to the neurons in this system (Harding et al., 1977; Monti Graziadei and Graziadei, 1979). We have found that a commercially available antiserum to keratins, the class of intermediate filaments characteristic of epithelial cells (e.g., Moll et al., 1982), specifically reacts with basal cells—and not the neurons or supporting cells—within the OE of all species so far tested (rat, man, and mouse). In postnatal mouse OE, keratin staining is limited to a single row of cells adjacent to the basement membrane (Figure 1A). In 15 day mouse embryos, this antiserum stains a large number of cells, many of which appear to span the entire width of the developing OE (Figure 1C). This is consistent with the findings of Smart (1971), who has shown that the nuclei

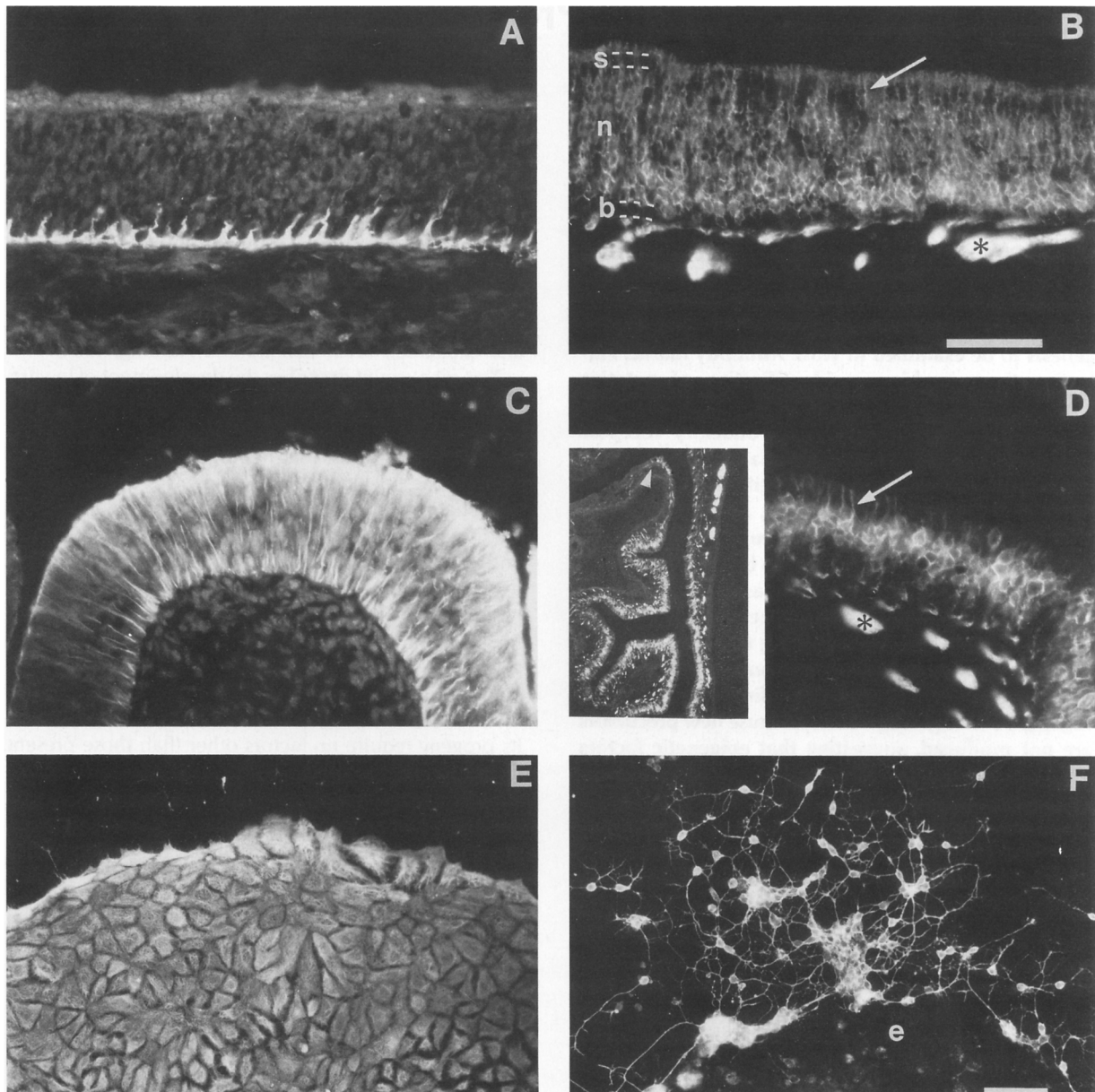


Figure 1. Immunocytochemistry of Basal Cells and Olfactory Receptor Neurons In Vivo and In Vitro

OE tissue and cultures were prepared as described in Experimental Procedures. (A), (C), and (E) show staining with rabbit antiserum to keratins in postnatal mouse OE, embryonic (E15) OE, and 36 hr OE cultures, respectively. (B), (D), and (F) show staining with AG1 monoclonal anti-N-CAM in the same tissues. In postnatal OE, rabbit antiserum to keratins stains the layer of basal cells immediately adjacent to the basement membrane (A); short processes of some of these cells can be seen extending into more apical regions of the epithelium. In OE of the same age, monoclonal anti-N-CAM (B) exclusively stains neurons in the extensive receptor neuron layer (n), but does not stain cells in the basal cell layer (b) or the luminal sustentacular cell layer (s). N-CAM is present on the entire surface of olfactory receptor neurons: the apical dendrites (arrow) and the bundles of axons (asterisk) of the receptor neurons are both brightly stained. In E15 OE in situ, anti-keratins antiserum stains about half of the cells (C; see Table 1 also), many of which extend from the basal to the apical surface. This staining pattern is consistent with the observation that, in the OE at this age, the nuclei of proliferating cells are present throughout the basal-apical extent of the epithelium and only later become restricted to the basal layer (see Smart, 1971). Monoclonal anti-N-CAM stains neurons in the developing olfactory receptor neuron layer in E15 OE (D); again the apical dendrites (arrow) and bundles of axons (asterisk) exiting the OE in the underlying stroma are stained. The anti-N-CAM antibody also clearly demarcates olfactory from nearby respiratory epithelium (D, inset): The arrowhead on the left side of the low magnification inset points to the junction between the anterior, unstained, respiratory epithelium and the posterior, stained, OE of the turbinates in the lateral regions of the developing nasal cavity. On the right side of the inset, across from the turbinates, is the posterior septum; this part of the septum is also covered by OE, and the neurons within it are stained as well. Sheets of cells with epithelial morphology are stained by the antiserum to keratins in OE cultures (E); this staining pattern is filamentous, consistent with this antibody recognizing the class of intermediate filaments (keratins) that is characteristic of basal cells. In (F), neurons can be seen adjacent to an epithelial sheet (e) in an OE culture. The neuronal cell bodies and neurites are stained with the monoclonal anti-N-CAM antibody, whereas cells of the epithelial sheet cannot be seen because they do not stain with this antibody. Bars, 50 μ M. Bar in (B) also applies to (A), (C), and (D); bar in (F) also applies to (E).

of proliferating cells are present throughout the OE at this stage in development and only later become restricted to the basal layer.

The olfactory receptor neuron is the only type of neuron in the OE. To identify this cell type we have used a monoclonal antibody that recognizes a cytoplasmic determinant common to the 140 and 180 kd forms of the neural cell adhesion molecule (N-CAM) polypeptide (DiFiglia et al., submitted). In postnatal mouse OE, this antibody stains the cell bodies and apical dendrites of olfactory receptor neurons in the extensive receptor neuron layer (Figure 1B, arrow). The bundles of receptor neuron axons exiting the epithelium in the submucosa are also stained (Figure 1B, asterisk). Cell bodies in the luminal sustentacular (supporting) cell layer (Figure 1B, s) do not stain with this antibody, nor do cells in the basal cell layer (Figure 1B, b). Similar findings have also been reported recently by Miragall et al. (1988). In embryonic OE, this antibody recognizes neurons in the developing receptor neuron layer; these cells have the characteristic apical dendrites of olfactory receptor neurons (Figure 1D, arrow). Staining with this antibody also clearly demarcates the neural, olfactory epithelium of the turbinate region and posterior septum, from the more anterior, nonneural, respiratory epithelium of the nasal cavity (Figure 1D, inset).

To optimize conditions for obtaining neurogenesis in vitro, we cultured OE from late gestation (E14 and E15) mouse embryos. At this age, the major features of the olfactory system are already present. Structures of the nasal cavity such as turbinates and septum are formed; these morphological landmarks make tissue dissections easily reproducible. Contacts between olfactory receptor neurons and olfactory bulb are present, and a few of these neurons already express olfactory marker protein (OMP), a cytoplasmic protein characteristic of mature receptor neurons (Farbman and Margolis, 1980; Monti Graziadei et al., 1980). Thus, we could be confident that the turbinate regions we dissected for our cultures were lined with olfactory, rather than respiratory, epithelium because they contained mature OMP-expressing neurons (data not shown) as well as N-CAM-expressing neurons (Figure 1D). In addition, a large proportion of total cells in the OE are proliferating at this age (Smart, 1971; Cuschieri and Bannister, 1975), predicting that in pure OE cultures the percentage of proliferating neuronal precursor cells should be relatively high.

To obtain pure OE cultures, we separated OE from its underlying mesenchymal stroma and cultured the purified epithelium in isolation (see Experimental Procedures). Since much of the mesenchyme of the head is believed to be of neural crest origin (Le Douarin, 1982), removal of stroma eliminated the possibility that any neurons generated in our cultures might be the progeny of contaminating neural crest cells. The supporting or "Schwann" cells of the olfactory nerve are also present in the stroma underlying the OE, so removing this tissue eliminated these cells as potential contaminants of our cultures as well.

When pieces of purified OE were explanted into cul-

ture, two major cell types were present. One of these had an epithelial morphology and grew in sheets of closely contacting cells. Filaments within these cells stained with the antiserum to keratins (Figure 1E), suggesting that these cultured "epithelial cells" are the in vitro equivalents of basal cells of the OE. The second major cell type comprised the neurons, which were present in profusion around the sheets of epithelial cells after about a day of culture. These neurons, but not the sheets of epithelial cells, were stained by the monoclonal antibody to N-CAM that stains olfactory receptor neurons in vivo (Figure 1F).

Thus, we could use antibody markers to detect both putative neuroepithelial stem cells (anti-keratin antiserum; basal cells) and differentiated neurons (monoclonal anti-N-CAM antibody; olfactory receptor neurons) in this culture system. A third cell type, a migratory, round, nonepithelial cell that appears at early times in these cultures, is not stained by either of these antibodies and is described below.

Lowering External Calcium in Olfactory Epithelium Cultures Allows Three Distinct Cell Types to Be Identified

When purified OE was placed into culture and observed over the course of 2 days, two morphological features were noted. The cultures contained flat epithelial sheets, and at the edges and lying on top of these sheets there were clusters of small, round cells (Figure 2A). The appearance of neurites emerging from some cells in the clusters suggested that they consisted, at least in part, of neurons (Figure 2A, arrow). Furthermore, the clustering of these cells at the edges of the epithelial sheets suggested that they were attempting to migrate off of the epithelium, but were unable to do so in the culture conditions employed.

One explanation for the inability of these cells to migrate off the epithelial sheet was the possibility that their adhesion to the culture substratum might not be strong enough to overcome their adhesion to one another. We reasoned that calcium-dependent adhesion processes might be an important component of cell-cell adhesion in this system and therefore tested the effect of lowering the concentration of calcium in our culture medium to 0.1 mM, approximately 20-fold less than the concentration in normal cell culture media. At this concentration, adherens junctions between epithelial cells are significantly disrupted (e.g., Duden and Franke, 1988).

When cultures grown in low calcium medium (LCM) were observed over the course of 2 days, dramatic differences in cell migration occurred, with the result that all of the cells in these cultures could now be much more easily observed. At 16 hr in LCM, loosely aggregated clumps of small, round cells could be seen migrating away from the attaching epithelial sheet (Figure 2B). By 33 hr, these cells were no longer apparent. Instead, in the regions surrounding the epithelial sheets there were many neurons extending fine, branching neurites (Figure 2C). The transient appearance of the population of migratory "round cells," and the subsequent appearance

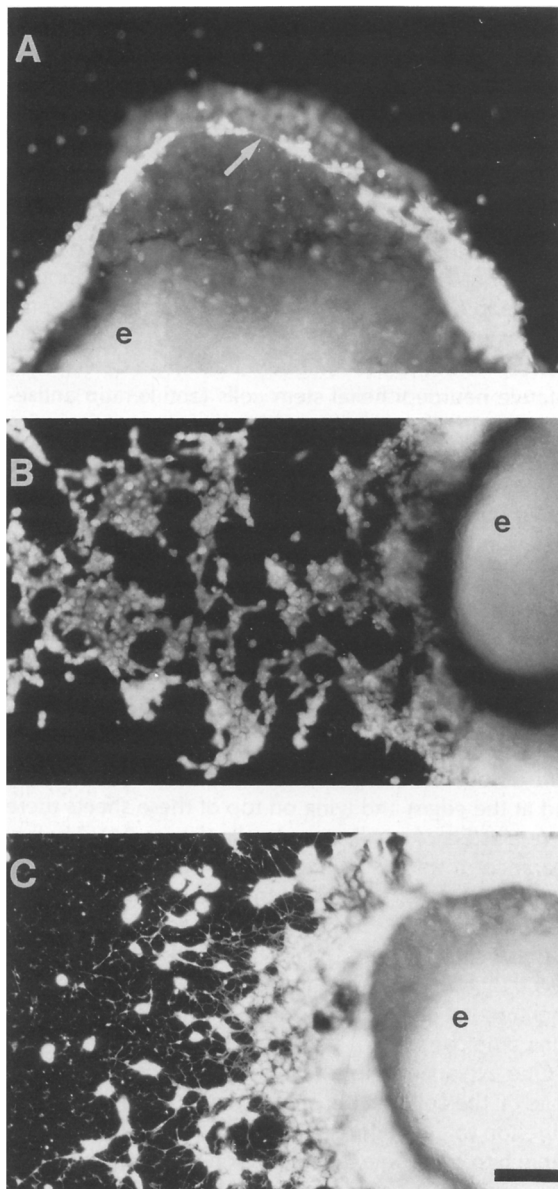


Figure 2. Cell Migration and Neuronal Differentiation in Olfactory Epithelium Cultures Grown in Low or Normal Calcium Medium. Cultures were grown in complete serum-free medium made with either normal or low calcium concentrations, as described in Experimental Procedures. At 16 or 33 hr after plating, cultures were rinsed with Hank's balanced salt solution (HBSS), then placed into 1 ml of a 30 μ M solution of carboxyfluorescein diacetate succinimidyl ester (Molecular Probes) made up in HBSS (Bronner-Fraser, 1985). Cultures were then incubated for 30 min at 37°C, rinsed twice in HBSS, and fixed immediately in 3.7% formaldehyde. (A) shows a culture grown in normal calcium medium for 16 hr. In this micrograph the epithelial sheet (e), which is fluorescent, is out of the plane of focus, which was chosen to show the bundles of round cells lying on top of the epithelial sheet. Processes extending between these bundles of cells (arrow) suggest that they contain at least some neurons. At 16 hr in LCM (B), there is extensive migration of loosely aggregated clumps of small, round cells away from the attaching epithelial sheet. A day later, neurons have extended a dense meshwork of fine, branched neurites in this region (C). The photograph in (C) is overexposed to show these neurites. Bar, 50 μ M. Bar in (C) also applies to (A) and (B).

of neurons in the regions to which the round cells had migrated, suggested that these cells were either precursors of neurons or neurons that had not yet extended neurites in culture.

The LCM used in these cultures did not noticeably decrease cell viability, as all of these cells were readily stained with carboxyfluorescein diacetate succinimidyl ester (Figure 2). (This dye diffuses readily into cells, but becomes fluorescent only when acted upon by cytoplasmic esterases; thus it only stains living cells.) Furthermore, the proportions of neurons and "basal" epithelial cells in these cultures were not affected by the lowered calcium condition. To determine this, OE was cultured in either normal or low calcium medium and at various times after plating, the cultures were dissociated into single cells and examined by anti-N-CAM and anti-keratin immunocytochemistry. Table 1 shows that, at any given time, the relative percentages of basal epithelial cells and neurons are essentially the same for cultures grown in low versus normal calcium conditions. Although the relative size of the neuronal population increases over time, this is observed in both conditions, indicating that epithelial cell growth and neuronal differentiation are proceeding in a similar manner. At early times in culture there are also cells that are negative for staining with either antibody, and the size of this keratin⁻, N-CAM⁻ population is essentially the same in both low and normal calcium. Because the enhanced migration of cells in LCM made it much easier to identify and count the cell types that emerged in these cultures, this medium was adopted for all quantitative studies of neurogenesis described below.

Neurogenesis Occurs in Cultured Olfactory Epithelium

To determine whether neurogenesis occurs in cultured OE, cells were exposed to [³H]thymidine for a short time early in the culture period, then allowed to grow for another day. When these cultures were processed for autoradiography, silver grains were present over the nuclei of cells with the morphological appearance of neurons (i.e., cells bearing neurites with visible growth cones). To confirm the identification of the neurite-bearing cells as neurons, they were stained with an antiserum to GAP-43 (Benowitz et al., 1988), a neuron-specific protein whose expression is correlated with neuronal development and regeneration (Basi et al., 1987; Benowitz and Routtenberg, 1987; Meiri et al., 1988). This anti-GAP-43 antiserum stained the neurite-bearing cells, but not the basal epithelial cells, in these cultures. In experiments combining [³H]thymidine autoradiography and GAP-43 immunocytochemistry, many GAP-43⁺ neurons were labeled with silver grains (data not shown). This confirmed that these labeled neurite-bearing cells were the neuronal progeny of a precursor that proliferates *in vitro*.

A Proliferating Nonepithelial Cell Population Divides Once during the Culture Period

Two types of quantitative experiments were used to

Table 1. Relative Sizes of Cell Populations in Olfactory Epithelium Cultures

Hours in Culture	Culture Condition	% Keratin ⁺ Cells (Basal Epithelial Cells)	% N-CAM ⁺ Cells (Neurons)
0	NA ^a	54 ± 2.2	29 ± 1.5
13	Low calcium	62 ± 6.1	28 ± 2.2
	Normal calcium	60 ± 4.1	27 ± 0.9
23	Low calcium	65 ± 2.1	27 ± 1.1
	Normal calcium	66 ± 5.3	25 ± 0.2
38	Low calcium	57 ± 3.1	37 ± 0.8
	Normal calcium	64 ± 1.3	32 ± 0.6

OE was purified and placed into culture in the normal manner, in either normal or low calcium medium, as described in Experimental Procedures. At the indicated times after plating, cultures were dissociated into single cells and cells were allowed to settle onto activated, aminopropyl silane-treated slides. Two slides were prepared from each of duplicate or triplicate cultures for each time point and culture condition. Slides were fixed and processed for immunocytochemistry using either the antiserum to keratins or the AG1 monoclonal anti-N-CAM. The percentage of cells that were stained with a given antibody was determined for each slide by counting cells under phase-contrast and epifluorescence optics. A minimum of 200 cells in 20 randomly chosen microscope fields were counted for each slide, and the values obtained for slides from duplicate or triplicate cultures were averaged. Values shown are the mean and range for each determination.

Absolute numbers of cells are not given in Table 1 for practical reasons. The data in this experiment, as in all experiments reported in this paper, were collected from individual cultures consisting of explanted pieces of OE. These cultures were always seeded with similar amounts of tissue, but there was no way to determine a priori the exact number of cells in a culture because the tissue had not been dissociated into a single-cell suspension. Consequently, it would not have been valid to draw conclusions from differences in cell numbers seen at later stages.

^a NA, not applicable. Freshly purified epithelium was dissociated for these determinations.

characterize the process of neurogenesis in these cultures. To determine which cells were proliferating at a given time in culture, separate, identical cultures were exposed to [³H]thymidine for a 2 hr period at various times and then fixed (pulse-fix paradigm). Following autoradiographic processing, the percentage of a given population of cells that was labeled was determined by counting the cells with silver grains over their nuclei. This labeling index reveals the percentage of cells that were in the phase of DNA synthesis at the time of [³H]thymidine administration.

Pulse-chase labeling experiments were done to identify the progeny of proliferating cells. In these experiments, identical cultures were pulsed with [³H]thymidine for 2 hr shortly after plating (10–12 hr in culture). [³H]thymidine was then removed, and cultures were refed with medium containing an excess of cold thymidine. At various times thereafter, cultures were fixed and processed for autoradiography. In this paradigm, the labeling index is the percentage of cells of a given type that have acquired [³H]thymidine; these cells are the progeny of dividing cells labeled in the initial pulse.

In determining labeling indices, we were particularly interested in the proliferative behavior of the migratory round cells seen at early times in these cultures because neurons always appeared in precisely those regions to which these cells had migrated. These cells were therefore obvious candidates for neuronal precursors. Accordingly, pulse-chase and pulse-fix labeling indices for the total nonepithelial population (i.e., all cells not belonging to the epithelial sheets, including both round cells and neurons) in these cultures were determined over the course of 2 days. Pulse-fix analysis shows that the nonepithelial population is initially capable of proliferating, but gradually ceases to synthesize DNA af-

ter a day in culture (Figure 3A, dashed line). Pulse-chase analysis shows that the percentage of nonepithelial cells accumulating [³H]thymidine after an initial pulse increases over time, from approximately 9% to 17%, and then plateaus (Figure 3A, solid line). This suggests a single round of division in these cells.

A separate analysis was performed to characterize the generation of neurons specifically. The labeling index for neurons (defined as cells bearing neurites >1 cell diameter in length) was determined in both pulse-fix and pulse-chase experiments (Figure 3B). The pulse-fix experiment shows that neurons are not a dividing cell population; they essentially never take up [³H]thymidine in a 2 hr pulse (Figure 3B, dashed line). This indicates that all of the [³H]thymidine uptake in the total nonepithelial population (Figure 3A) must be into the migratory round cells. However, neurons are derived from a cell population that synthesizes DNA early on in the culture period, since the pulse-chase experiment shows that, after an initial pulse of [³H]thymidine, the number of neurons that accumulate [³H]thymidine steadily increases over time (Figure 3B, solid line).

The presence of the plateau in the pulse-chase labeling index in Figure 3A (solid line) indicates that the round cells are a population that is withdrawing from the cell cycle. This is also clearly indicated by the results of the pulse-fix analysis for these cells (Figure 3A, dashed line). That this withdrawal is a consequence of the progeny of the round cells differentiating into neurons is suggested by the steady increase in [³H]thymidine label appearing in differentiated neurons over this period (Figure 3B, solid line). Comparison of Figures 3A with 3B shows that the withdrawal of the round cells from the mitotic cycle and the appearance of [³H]thymidine in differentiated neurons occur over similar time courses.

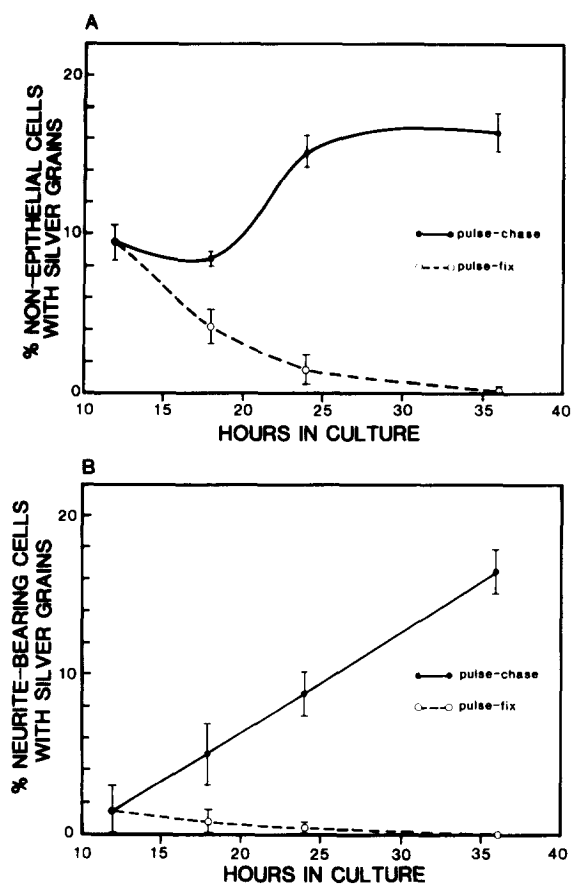


Figure 3. $[^3\text{H}]$ Thymidine Uptake Analysis of Nonepithelial Cell Proliferation and Neuron Generation in Olfactory Epithelium Cultures

For pulse-fix analysis, cultures were pulsed with $[^3\text{H}]$ thymidine ($5 \mu\text{Ci/ml}$, 80 Ci/mmol) for 2 hr at 6 hr intervals beginning 10 hr after plating and then fixed immediately. For pulse-chase analysis, cultures were all given an identical pulse of $[^3\text{H}]$ thymidine from 10 to 12 hr after plating; the culture medium was then replaced with growth medium containing an excess of cold thymidine, and duplicate cultures were fixed thereafter at 6 hr intervals. Cultures were processed for autoradiography as described in Experimental Procedures, and the percentages of cells with silver grains over their nuclei were determined. Values represent the mean and range of determinations made on duplicate cultures. Data for both graphs came from the same experiment. (A) Labeling indices for all nonepithelial cells. This population includes both round cells and neurons, but excludes basal cells of the epithelial sheet. (B) Labeling indices for neurons only. Neurons are defined here as nonepithelial cells bearing neurites >1 cell diameter in length. For further discussion of these graphs, see text.

The Neuronal Precursor Is an N-CAM⁻ Cell That Gives Rise to Two N-CAM⁺ Neurons

The experiments illustrated in Figure 3 suggest that the nonepithelial, migratory round cells are the neuronal precursors in these cultures. Accordingly, it seemed possible that they might express some of the same cell surface antigens expressed by the neurons. We knew that N-CAM was expressed by the many neurons present in older (24–48 hr) cultures. To determine whether the putative precursors also expressed N-CAM, we stained

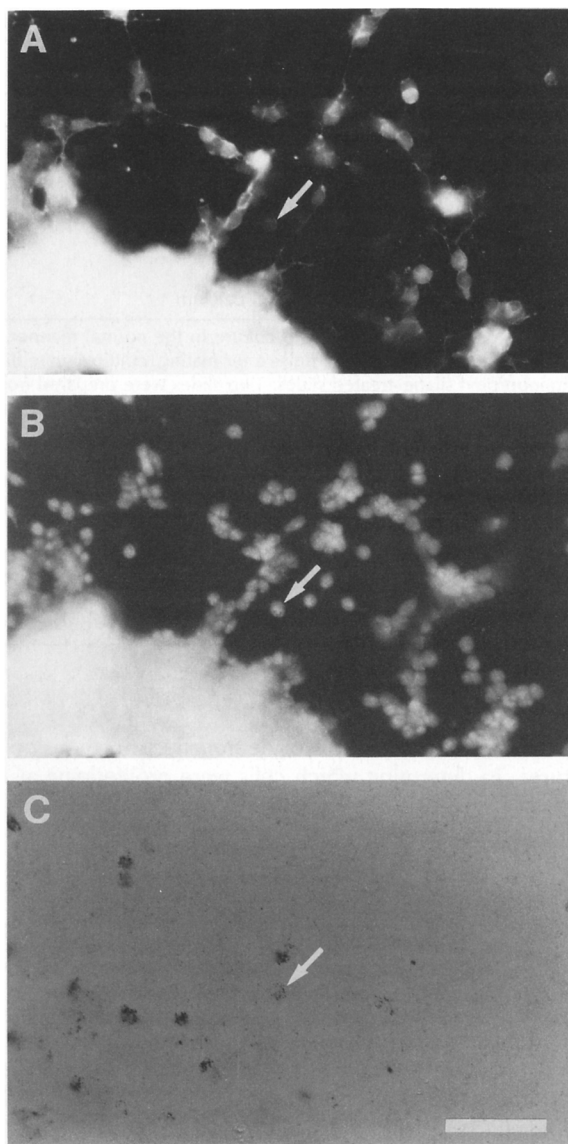


Figure 4. The Proliferating Neuronal Precursor Is an N-CAM⁻ Cell. Cultures were pulsed with $[^3\text{H}]$ thymidine ($5 \mu\text{Ci/ml}$, 80 Ci/mmol) for 2 hr at 10 hr after plating. Some cultures were fixed immediately, and the rest were re-fed with medium containing an excess of cold thymidine. At 6 hr intervals, duplicate cultures were fixed, stained with monoclonal anti-N-CAM, and processed for autoradiography as described in Experimental Procedures. (A)–(C) are pictures of the same field, taken from a culture that was fixed at 12 hr after plating, immediately after receiving the pulse of $[^3\text{H}]$ thymidine. (A) Fluorescence micrograph showing cells emerging from a 12 hr epithelial explant. Both N-CAM⁺ and N-CAM⁻ (arrow) cells are migrating away from the explant. The explant is incompletely attached and still contains many preexisting N-CAM⁺ neurons that are out of the plane of focus; hence it appears as a brightly stained mass in the lower left quadrant of the field. (B) Nuclear staining with Hoechst; same field. The nuclei of many N-CAM⁻ migrating round cells can now be seen. The arrow points to the same cell as in (A). (C) Bright-field micrograph showing silver grains over the nuclei of cells in this field. The arrow points to the N-CAM⁻ cell seen in (A) and (B); it is labeled with $[^3\text{H}]$ thymidine. Other clusters of silver grains, also over the nuclei of N-CAM⁻ migrating cells, can be seen as well. Bar, 50 μm .

cultures shortly after plating (12 hr), when many loosely aggregated clumps of these cells could be seen migrating away from the attaching epithelial explant. Surprisingly, the antibody to N-CAM labeled only a subpopulation of round cells.

To test whether the presence or absence of N-CAM expression reflected the proliferative state of this subpopulation of round cells, we performed pulse-fix and pulse-chase [^3H]thymidine uptake experiments in combination with anti-N-CAM immunocytochemistry. Figures 4A–4C are all photographs of the same field in a culture that was pulsed with [^3H]thymidine from 10–12 hr after plating, then fixed immediately and processed for N-CAM immunocytochemistry and autoradiography. This figure shows that there are two distinct classes of the migratory round cells: those that take up [^3H]thymidine and are N-CAM $^-$ and those that are N-CAM $^+$ but do not take up [^3H]thymidine. We observed that N-CAM $^+$ cells were usually found in the same clumps as N-CAM $^-$ cells. Furthermore, close inspection revealed that the N-CAM $^+$ cells in the clumps often had short neurites that were only apparent with immunofluorescent staining (Figure 4A). These observations suggested that while neuronal precursor cells that are actively synthesizing DNA do not express N-CAM, their neuronal progeny, which have withdrawn from the mitotic cycle, do express N-CAM. This in turn would mean that expression of N-CAM is an early biochemical marker for neuronal differentiation of these cells.

This idea is supported by the pulse-chase analysis illustrated in Figure 5. In this analysis, the percentage of [^3H]thymidine-labeled nonepithelial cells that were also stained with the antibody to N-CAM was determined at various times following a 2 hr pulse early in the culture period. At the time of the initial pulse, only cells in the N-CAM $^-$ population actively incorporate [^3H]thymidine. Over the course of 2 days in culture, however, this [^3H]thymidine label moves from being entirely in the N-CAM $^-$ (precursor) population to being predominantly in the N-CAM $^+$ population (essentially all of which are neurite-bearing cells). The delay between incorporation of [^3H]thymidine and first expression of N-CAM suggests that neuronal precursor cells must complete the mitotic cycle before they can express N-CAM. Indeed, nonepithelial cells in these cultures that are labeled by an early 2 hr [^3H]thymidine pulse undergo a wave of division lasting from 6 to 14 hr after the pulse (Figure 3A, solid line; 18–24 hr in culture), whereas such cells begin to express N-CAM only between 6 and 24 hr following the pulse (Figure 5; 18–36 hr in culture).

These data also suggest that the majority of neuronal precursors divide to give rise to two N-CAM $^+$ daughter neurons. We reasoned that, if this was true, it should be possible in later cultures to see pairs of N-CAM $^+$ sister neurons derived from a precursor cell that took up [^3H]thymidine during an early pulse label. Such events frequently occur, as illustrated in Figure 6. The two N-CAM $^+$ neurons seen in Figure 6A are from a 36 hr culture that had been pulsed with [^3H]thymidine between 10 and 12 hr after plating. These two neurons,

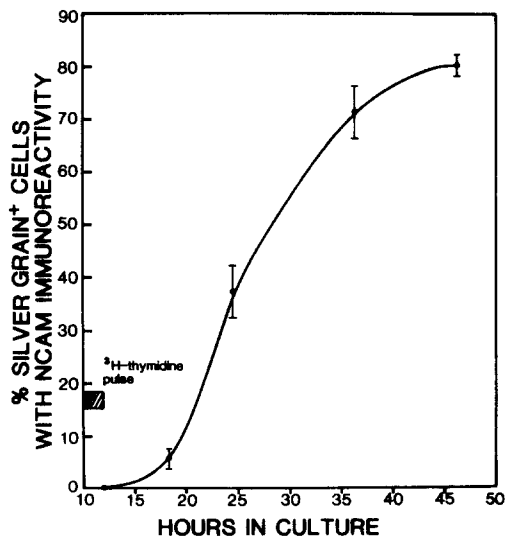


Figure 5. The N-CAM $^-$ Neuronal Precursor Divides to Give Rise to Two N-CAM $^+$ Neurons

Quantitative analysis of the combined pulse-chase [^3H]thymidine uptake/N-CAM immunocytochemistry experiment illustrated in Figure 4. The percentage of [^3H]thymidine-labeled migratory, nonepithelial cells that were also stained with the antibody to N-CAM was determined at 6 hr intervals following the initial 2 hr pulse. Values represent the mean and range of duplicate cultures. For discussion of graph, see text.

strikingly similar in morphology, have nearly identical densities of silver grains over their nuclei (Figure 6B). This fact, together with their proximity, suggests that they are derived from a common precursor.

Continued Neurogenesis Fails to Occur In Vitro

In vivo, the OE maintains the ability to generate neurons throughout life (Graziadei and Monti Graziadei, 1978). Accordingly, it was thought initially that continual generation of neurons would also be seen in OE cultures. Instead, neurogenesis ceases rapidly. As Figure 3A shows, the pulse labeling index over nonepithelial cells declines to near zero by 36 hr in culture. The data presented in Figures 3, 4, and 5 suggest that the cessation of neurogenesis reflects the fact that division of N-CAM $^-$ round cells is followed by the differentiation of both daughter cells into postmitotic neurons. No cell divisions in these cultures appear to generate new dividing neuronal precursor cells. With time in culture, not only do the round cells disappear, but the differentiated neurons abruptly begin to die, and by 7 days are no longer detectable. The sheet of keratin $^+$ epithelial cells, in contrast, remains viable and proliferative for at least 14 days in vitro, the longest time assayed (data not shown). Yet even with long-term cultivation of these basal cells, neither neurons nor other nonepithelial cell types reappear. This is the case regardless of whether these cells are cultured in the presence of normal or low concentrations of calcium, in defined medium, or in the presence of serum (data not shown).

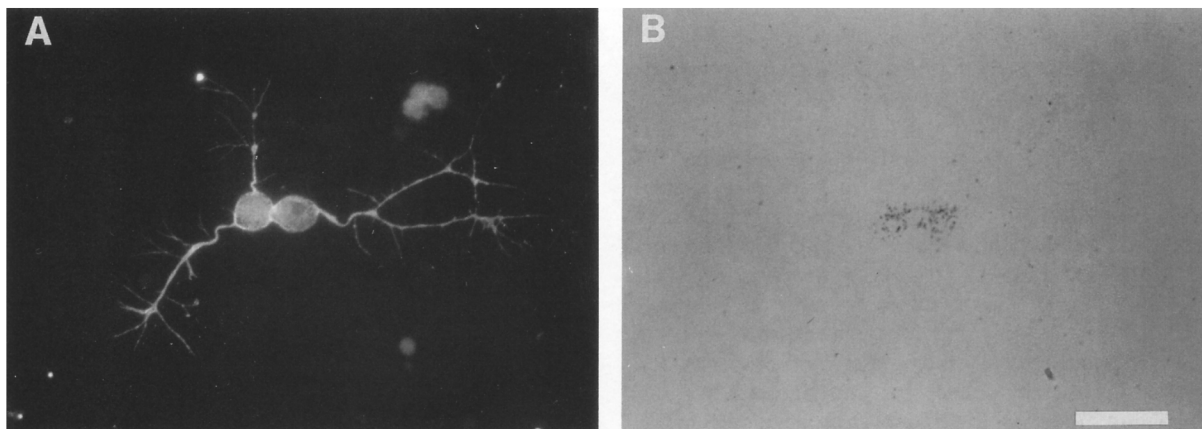


Figure 6. N-CAM⁺ Sister Neurons Generated in Olfactory Epithelium Cultures
Fluorescence (A) and bright-field (B) micrographs of two N-CAM⁺ sister neurons in a 36 hr culture, taken from the pulse-chase [³H]thymidine uptake/immunocytochemistry experiment illustrated in Figures 4 and 5. Bar, 25 μ m. For explanation, see text.

Discussion

The Immediate Neuronal Precursor Is Distinct from the Basal Epithelial Cell

In vivo studies of neurogenesis and neuronal regeneration in the OE suggest, albeit indirectly, that basal cells are the self-renewing neuronal progenitors of this tissue (Graziadei, 1973; Harding et al., 1977; Monti Graziadei and Graziadei, 1979). In cultures of OE, cells with the characteristics of basal cells (keratin⁺, N-CAM⁻, epithelial morphology) occur in abundance (e.g., Figure 1E; Table 1), but the production of neurons by these cells in vitro was not observed.

Instead, several lines of evidence indicate that the immediate precursor of neurons is a round, keratin⁻, N-CAM⁻ cell that, under appropriate culture conditions, migrates away from the keratin⁺ epithelial sheet and then divides to give rise to two daughter neurons. First, the round cells are the proliferating cells that are found in closest proximity to newly generated neurons. This is particularly dramatic in LCM, in which the round cells migrate away from the keratin⁺ epithelial cells (Figure 2). Second, neurons always differentiate only in the vicinity of round cells, even when the latter have migrated very asymmetrically—e.g., in only one direction away from the epithelium—or when the epithelial sheet itself becomes detached and is lost within the first 12 hr of culture (both were common occurrences). Third, the keratin⁻, N-CAM⁻ round cells divide only once during the culture period and then disappear with a time course that closely corresponds to that of the production of new neurons (Figure 3). Particularly striking is the degree to which the generation of N-CAM⁺ cells from N-CAM⁻ precursors parallels the time course of round cell division (compare Figure 3A with Figure 5).

Interestingly, cells that are morphologically distinct from both basal cells and neurons have previously been described in the basal compartment of the OE ("globose" basal cells; see Graziadei and Monti Graziadei, 1979),

although these cells have never been specifically recognized as proliferating neuronal precursors. The data obtained in this study suggest that globose basal cells, or perhaps a subpopulation of globose basal cells, may be a dividing cell population and predict that these dividing cells will be found to have a keratin⁻, N-CAM⁻ phenotype.

Neurogenesis Occurs Efficiently In Vitro

The process of neurogenesis appears to be very efficient in this system, as two lines of evidence suggest that the majority of neuronal precursors (round cells) present in these cultures give rise to neurons:

First, the data suggest that most neuronal precursors are probably mitotically active. At 12 hr in culture, the [³H]thymidine pulse labeling index over total nonepithelial cells is approximately 10% (Figure 3A). This population of cells includes both neuronal precursors (N-CAM⁻ round cells) and neurons (N-CAM⁺), the relative proportions of which may be estimated from Table 1 to be approximately 1:3 at this time in culture. [Taking into account the fact that the keratin⁺ and N-CAM⁺ populations are nonoverlapping (Figure 1), the proportions after 13 hr in culture are approximately 62%:28%:10% keratin⁺:N-CAM⁺:keratin⁻, N-CAM⁻. Thus, the ratio of N-CAM⁺:N-CAM⁻ cells in the nonepithelial population is 28%:10%, or about 3:1.] Since neurons themselves do not divide (Figure 3B), but instead only N-CAM⁻ round cells incorporate [³H]thymidine (Figure 4), the 10% labeling index over total nonepithelial cells actually represents a labeling index over N-CAM⁻ round cells of 40% (4 times higher, because the N-CAM⁻ cells are a fourth of the population). [³H]thymidine pulse labeling labels only those cells in the phase of DNA synthesis, and since this phase accounts for only part—typically less than half—of the entire cell cycle, it is likely that the majority of N-CAM⁻ round cells are dividing cells.

Second, most of the progeny of these precursors be-

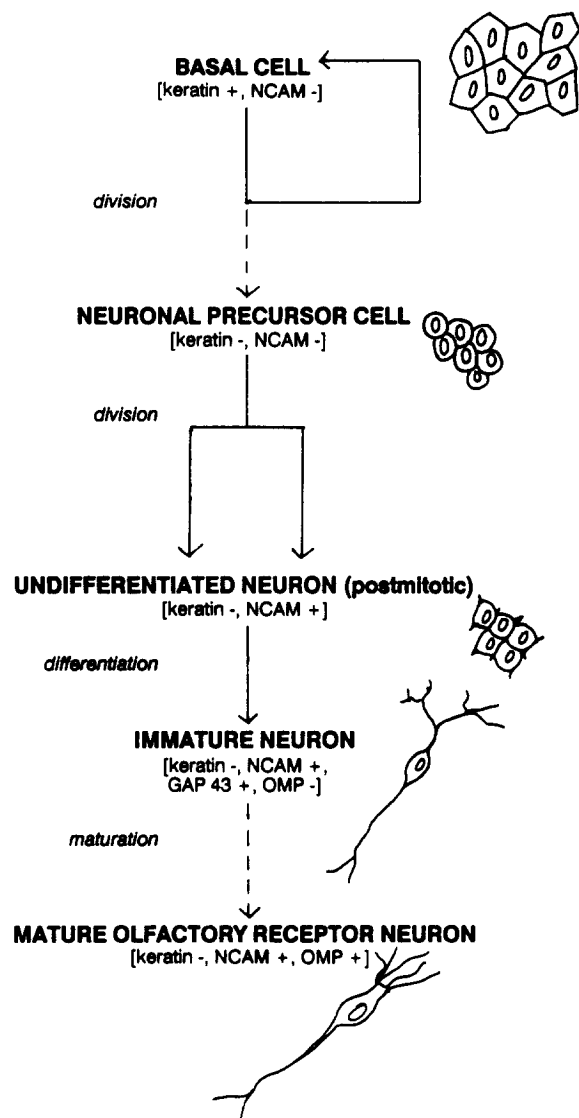


Figure 7. Neurogenesis in the Olfactory Epithelium: A Proposed Sequence of Events

A proposed series of steps in proliferation and differentiation leading to the production of olfactory receptor neurons is diagrammed. Solid arrows indicate steps whose existence is directly supported by the observations in this manuscript. Dashed arrows indicate steps whose occurrence has been previously suggested by *in vivo* studies. For further discussion, see text.

come N-CAM⁺ neurons. This is illustrated in Figure 5. Over 80% of the [³H]thymidine label taken up by the N-CAM⁻ round cell population at 12 hr in culture moves into the N-CAM⁺ neuronal population by 2 days. Thus, since most neuronal precursors divide, and since the majority of their progeny are neurons, neurogenesis occurs efficiently.

Is the Neuronal Precursor a Unipotential Cell?

The simplest explanation for the fact that over 80% of the detectable progeny of labeled round cells become neurons (Figure 5) is that the immediate neuronal precursor follows a simple lineage program, dividing to give rise to two N-CAM⁺ daughter neurons. Although it

is possible that such a result could be obtained if each precursor gave rise to one neuron and one other (non-neuronal) cell, followed rapidly by the selective death of the nonneuronal cell, we do not believe that this occurs. First, we frequently observe what appear to be pairs of sister neurons (closely apposed N-CAM⁺ neurons with similar morphologies and degrees of [³H]thymidine labeling) in these cultures (Figure 6), reinforcing the notion that the proposed lineage program is correct. Second, selective cell death does not seem to be a problem. In Figure 3A, the labeling index for round cells should not double if half of the progeny of the dividing cells selectively die. Of course, there remains the formal possibility that preexisting neurons also die in just the right numbers to counterbalance the selective loss of newly generated nonneuronal cells, thereby producing the apparent doubling in labeling index seen in Figure 3A. However, a large number of neurons would need to die in order for this to occur. This in turn would require the death of a large number of epithelial cells in order to maintain the proportions shown in Table 1. None of our observations suggest that this occurs over the time course in which these experiments take place.

Finally, we see no evidence that the round cells give rise to cells other than neurons. At 2 days, we observe essentially two cell types in these cultures – basal epithelial cells and neurons – and our quantitative immunocytochemical analysis of the relative sizes of these cell populations at about this time in culture confirms this observation (Table 1). In addition, we do not observe cells other than N-CAM⁺ neurons arising in the areas to which round cells have migrated. No evidence has been obtained that these precursors also give rise to the supporting or “sustentacular” cells of the OE, a cell type that is N-CAM⁻ *in vivo* (Figure 1, Miragall et al., 1988). Since these glia-like cells are themselves a proliferating cell type (Graziadei and Monti Graziadei, 1979; Graziadei, 1973), it may be that only a small number of them need to be generated during embryonic development. Consequently, their production *in vitro* by “neuronal” precursors might be missed. However, although this is a formal possibility, there are good reasons to believe that neurons and sustentacular cells simply are not related by lineage. Embryological studies suggest that sustentacular cells migrate into the OE from nonneural ectoderm (Klein and Graziadei, 1983). In addition, in the mature OE, cells expressing sustentacular cell-specific markers have been found within submucosal glands (Hempstead and Morgan, 1983). The possibility that these represent precursors of sustentacular cells is suggested by observations that cells derived from submucosal glands provide a source of sustentacular cells during OE regeneration (Mulvaney and Heist, 1971; Matulionis, 1976).

We conclude that *in vitro*, the immediate neuronal precursor in the OE follows a simple lineage program, dividing once to give rise to two N-CAM⁺ neurons. It is not possible, on the basis of these experiments, to determine whether this precursor divides only once *in vivo*; only experiments that trace the fates of individually labeled precursors *in vivo* can resolve this issue.

Neuronal Differentiation: A Multistage Process?

Observations of the process of neurogenesis in OE cultures suggest that neuronal differentiation is a multistage process. A proposed sequence of events is outlined in Figure 7.

These cultures contain cells that have been shown, on the basis of intermediate filament phenotype and morphology, to be the equivalents of basal cells in the OE, and we know that these basal cells proliferate in culture. [During the course of our ^3H]thymidine labeling experiments, we observed that many of the cells in the epithelial sheets were labeled. In fact, after 48 hr of continuous labeling with ^3H]thymidine, over 90% of these cultured basal cells were labeled, indicating that this is a highly proliferative cell type.] The data presented in this manuscript indicate that, if basal cells are the ultimate progenitors of olfactory receptor neurons—as suggested by *in vivo* studies (see discussion above)—then they give rise to neurons by way of an intermediate cell type, which divides as it migrates away from them. Although there has been no previous evidence of the existence of this immediate neuronal precursor in the OE, examples of analogous phenomena can be found in several regions of the developing central nervous system: small neurons of the area dentata of the hippocampal formation, the cerebellum, and the cochlear nuclei arise from precursors that migrate away from the germinal neuroepithelium and proliferate as they migrate (Miale and Sidman, 1961; Angevine, 1965; Altman and Das, 1966; Taber Pierce, 1967; reviewed in Angevine, 1970).

The fact that the immediate neuronal precursors give rise only to neurons in OE cultures suggests that these cells are already specialized for neuronal differentiation prior to their final mitosis. Indirect evidence for this specialization also comes from the recent discovery of a monoclonal antibody that stains the precursors and neurons, but not the basal cells of the epithelial sheet, in these cultures (A. L. Calof and W. D. Matthew, unpublished data). It is interesting that neuronal differentiation occurs in the absence of both the synaptic target tissue and the supporting stroma of this neuroepithelium. This suggests that these precursors might be programmed for a neuronal lineage and that their terminal differentiation into neurons is not under the control of epigenetic factors. However, the possibility that other cells present in these cultures might be providing signals required for neuronal differentiation cannot be excluded.

Maturation of these neurons into fully specialized olfactory receptor neurons presumably does require other factors, such as interactions with target tissue (olfactory bulb). While the neurons that arose in these cultures had extensive neurites and expressed two neuron-specific proteins (GAP-43 and N-CAM), they did not express OMP, a cytoplasmic protein characteristic of mature olfactory receptor neurons (Margolis, 1980), at a level detectable by immunocytochemistry (data not shown). Recent reports by Verhaagen and colleagues (Verhaagen et al., 1988, *Soc. Neurosci.*, abstract; Verhaagen et al., 1989) confirmed that OMP and GAP-43 are present in

mutually exclusive populations of cells in the developing and regenerating OE, with GAP-43 being expressed in newly generated, immature neurons and OMP arising later.

Regulation of Neurogenesis in the Olfactory Epithelium

In vivo, neurogenesis in the OE continues throughout life, whereas in cultures of this tissue, neurogenesis occurs for only a few days and then ceases. Neurogenesis ceases *in vitro* because existing keratin⁺, N-CAM⁺ neuronal precursors divide and differentiate efficiently, but no new precursors are produced to replace them.

In vivo studies suggest that cellular interactions regulate neurogenesis in the OE. When olfactory receptor neurons are induced to degenerate (e.g., by axotomy or by removal of their synaptic target, the olfactory bulb), mitotic activity in the epithelium increases; as newly produced cells differentiate into neurons, mitotic activity declines again (e.g., Camara and Harding, 1984). This suggests that the production of new olfactory receptor neurons from their precursors is regulated by the presence of previously differentiated olfactory receptor neurons. Such cell-specific regulation of neuronal production has also been proposed in the retina (Reh and Tully, 1986; Reh, 1987). It is unlikely, however, that the appearance of differentiated neurons accounts for the cessation of neurogenesis in OE cultures. As described above, neurons generated in these cultures survive less than a week (not surprising given that olfactory receptor neurons die *in vivo* if their connections with olfactory bulb are not maintained). Yet even after these neurons die, neurogenesis does not resume *in vitro*. Possibly there are other cellular interactions that are necessary for continual neurogenesis but are lacking *in vitro*. It is also possible that the cessation of neurogenesis *in vitro* reflects the absence of a required soluble factor, such as a polypeptide mitogen; studies to test this possibility are underway.

Interestingly, the phenomenon of cessation of neurogenesis *in vitro* has been alluded to by other investigators, who have reported that ^3H]thymidine administered on the first day of culture of some central nervous system tissues can give rise to labeling of a small fraction of neurons (e.g., Trenkner et al., 1984). Understanding why neurogenesis does not continue in these systems is complicated by the fact that neural tube-derived tissues normally stop generating neurons *in vivo*, so their *in vitro* behavior could reflect an acceleration of their normal developmental program rather than the absence of factors crucial for the maintenance of neurogenesis. Because the OE is not subject to such a developmental program, *in vitro* cessation of neurogenesis in this tissue probably reflects the latter alternative. Thus, the OE may provide a better model with which to define the substances or cellular interactions that are required to induce, maintain, or inhibit the generation of neurons.

Experimental Procedures

Animals

Random-bred CD-1 mice (Charles River) were naturally mated. The presence of a vaginal plug indicated successful mating, and the day of the vaginal plug was designated day 0 of pregnancy. For embryonic OE cultures and immunohistochemistry, embryos were taken at day 14 or 15 of pregnancy. Postnatal tissue for immunohistochemistry was from CD-1 mice that were 3–4 weeks of age at the time of sacrifice.

Materials

Tissue culture media and antibiotics were purchased from GIBCO-BRL. Laminin (purified from the EHS sarcoma) was the generous gift of Arthur Lander (Massachusetts Institute of Technology). Purified fibronectin (from human plasma) was purchased from New York Blood Center (New York, NY). Recombinant epidermal growth factor (EGF) was from Amgen. Glass coverslips (0.5 inch diameter, thickness 1) were obtained from Propper Mfg. (Brooklyn, NY), and plasticware was from Falcon. NTB2 emulsion, D-19 developer, and fixer were from Eastman Kodak. Fluorescent conjugates were from BRL (Texas red avidin), Tago (rhodamine-conjugated goat anti-mouse IgM and rhodamine-conjugated goat anti-rabbit IgG), Kirkegaard-Perry (fluorescein-conjugated goat anti-rabbit IgG), and Zymed (rhodamine-conjugated goat anti-mouse IgG). Reagents for alkaline-phosphatase immunohistochemistry were obtained from Vector, as were biotinylated secondary antibodies (e.g., biotinylated rabbit anti-sheep IgG). Reagents for IgG purification from ascites fluid were obtained from Bio-Rad, and biotinylation of IgG was accomplished using LC-NHS biotin and dimethylsulfoxide from Pierce. Unless specifically indicated, all other reagents were purchased from Sigma.

Tissue Culture

Dissection and Purification of Olfactory Epithelium

Embryos were dissected in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). OE was separated from underlying stroma according to Tyler and Pratt (1980). The turbinate regions were dissected out of the nasal cavity and incubated in ice-cold trypsin-pancreatin (Difco) solution (3%:1% in CMF-PBS) for 40 min. They were then transferred first to cold CMF-PBS containing 0.3% glucose, soybean trypsin inhibitor (250 µg/ml), and crystalline bovine serum albumin (BSA, 0.5 mg/ml; ICN), and then cold CMF-PBS plus only BSA. OE was purified away from stroma by trituration using a hand-held, flame-polished Pasteur pipette while visualizing the process under dark-field illumination with a transilluminating dissecting microscope. Pieces of purified OE were hand-picked and transferred through 2 changes of complete LCM (see below) before being plated onto glass coverslips in the wells of a 24-well culture dish.

Preparation of Substrata

Glass coverslips were cleaned by boiling in 1% HCl for 1 hr, rinsed twice in boiling distilled water, once in 100% ethanol, and dried. Cleaned coverslips were coated with a 1 mg/ml solution of poly-D-lysine hydrobromide (average MW ~100,000) overnight, washed in deionized distilled water, and sterilized by UV irradiation. Sterile, poly-D-lysine-coated coverslips were incubated overnight in a solution of 50 µg/ml laminin and 25 µg/ml fibronectin in CMF-PBS. After 5 washes in sterile CMF-PBS, coverslips were placed in the wells of a 24-well culture dish and covered with complete medium (see below). Pieces of purified OE were plated onto the coverslips.

Culture Media

Calcium-free Dulbecco's modified Eagle's medium (DME; high glucose formulation) was made from concentrated salt and vitamin mixtures obtained from GIBCO and supplemented to DME standard glucose, NaHCO₃, MgSO₄, and Fe(NO₃)₃·9H₂O concentrations using tissue culture grade reagents. To make LCM, 2 parts calcium-free DME were mixed with 1 part Ham's F12. The resulting mixture was 0.1 mM in calcium. Complete LCM consisted of the above mixture supplemented with crystalline BSA (500 µg/ml); insulin (10 ng/ml); transferrin (10 µg/ml); progesterone (20 nM); putrescine (100 µM); selenium (300 nM); and EGF (50 ng/ml) (Bot-

enstein and Sato, 1979; Grove and Pratt, 1984). For comparison experiments, normal calcium medium was made by supplementing the DME component of LCM to normal (2 mM) calcium concentrations using a concentrated CaCl₂ stock and mixing this with F12 in a 2:1 ratio as before. The resulting calcium concentration of normal calcium medium was 1.4 mM.

Cultures were incubated at 37°C in a 5% CO₂/95% air atmosphere.

Immunocytochemistry

Cultured Cells

Optimal fixation conditions and immunocytochemical procedures differed for different primary antibodies. For staining with rabbit antiserum to keratins (Dako rabbit polyclonal anti-keratin, wide spectrum screening; Dakopatts Z622), cultures were fixed in 5% acetic acid, 95% methanol at -20°C, then incubated overnight at 4°C in primary antiserum (1:400 dilution) followed by goat anti-rabbit fluorescein (1:50) or goat anti-rabbit rhodamine (1:200). For staining with monoclonal anti-N-CAM (AG1 hybridoma cell line; generously provided by M. Yamamoto, Eunice Kennedy Shriver Center, Waltham, MA), cultures were fixed at room temperature in acetone and stained with hybridoma supernatant followed by goat anti-mouse IgG rhodamine (1:30). For staining with the IgG fraction of sheep antiserum to GAP-43 (generous gift of L. Benowitz, MacLean Hospital, Belmont, MA), cultures were fixed in 3.7% formaldehyde in PBS. Staining with sheep anti-GAP-43 was performed with a 1:500 dilution of the reconstituted IgG fraction of the antiserum using published procedures (Benowitz et al., 1988) and visualized by rabbit anti-sheep biotin (1:200) followed by avidin-alkaline phosphatase (as specified in Vectastain ABC-AP kit). Negative controls for this staining were performed using exactly the same methods, except that a 25 µg/ml solution of sheep IgG (Sigma) was used in place of the primary antibody.

Tissue Sections

For staining with antiserum to keratins, embryos were fixed by immersion and postnatal mice by perfusion with 4% paraformaldehyde. Embryos or dissected postnatal turbinates were embedded in gelatin (Lallier and Bronner-Fraser, 1988) and cryostat-sectioned at 14 µm. Sections were permeabilized with Triton X-100 (0.2%) prior to staining and were visualized with the secondary antibodies listed above. To avoid cross-reactivity of secondary antisera with endogenous mouse immunoglobulin during staining with monoclonal anti-N-CAM, AG1 IgG was purified from ascites fluid by protein A affinity chromatography (Bio-Rad MAPS II system) and coupled to long-chain biotin (Pierce LC-NHS-biotin) according to the supplier's instructions. Biotinylated anti-N-CAM was visualized with Texas red avidin (1:1000). For anti-N-CAM staining, embryos and dissected postnatal turbinates were fixed by freezing in isopentane chilled on dry ice, then rehydrated by freeze-substitution, embedded in gelatin, and sectioned as above.

Analysis of Dissociated Cells

Cultures were rinsed in CMF-PBS, then incubated for 15 min in 1 ml of CMF-PBS containing 1 mg/ml trypsin (Sigma type III-S) and 1 mM EDTA. Enzymatic digestion was stopped by the addition of 250 µg of soybean trypsin inhibitor. Cells were pelleted for 10 min at 100 × g, resuspended to a single-cell suspension by extensive trituration in cold CMF-PBS, and plated for 1 hr at 4°C on glass slides that had been treated with 3-aminopropyl-triethoxysilane (Berger, 1986) and activated with 0.25% glutaraldehyde. Cells were then fixed onto slides by incubation in acetone for 5 min at room temperature. Immunocytochemistry was performed as described above for cultured cells. Cells were counted under phase-contrast and epifluorescence optics using a 40× objective. For each data point, dissociated cells from two cultures were counted and the mean and range were calculated. For each culture, a minimum of 200 cells in a minimum of 20 fields were scored.

Autoradiographic Analysis of Cell Proliferation

For pulse-fix analysis, cultures were incubated at designated times for 2 hr in 5 µCi/ml [³H]thymidine (80 Ci/mmol; New England Nuclear), then fixed prior to immunocytochemical processing. Follow-

ing incubation with secondary antibody, coverslips were dehydrated and reverse-mounted on microscope slides, dipped in NTB2 emulsion diluted 1:1 in water, exposed for 48 hr at -70°C , and developed in D-19 developer. Nuclei were stained with Hoechst 33258 (bisbenzimidazole, 1 $\mu\text{g}/\text{ml}$). For pulse-chase analysis, cultures were treated exactly as above, except that following the 2 hr incubation with [^3H]thymidine, cultures were re-fed with complete LCM containing 50 μM unlabeled thymidine and incubated for designated times prior to fixation and subsequent processing.

Quantitative analysis was performed by examining processed cultures with a 63 \times objective. Cells were scored for morphology, fluorescence, and the presence of silver grains over their nuclei. Each experiment was performed a minimum of 3 times; each figure shows data taken from a single experiment. For each data point, duplicate cultures were examined; for each culture, a minimum of 100 labeled cells in a minimum of 15 fields were scored.

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Note Added in Proof

The work cited as DiFiglia et al., submitted, is now in press: DiFiglia, M., Marshall, P., Covault, J., and Yamamoto, M. (1989). Ultrastructural localization of molecular subtypes of immunoreactive neural cell adhesion molecule (NCAM) in the adult rodent striatum. *J. Neurosci.*