Neurogenesis and Cell Death in Olfactory Epithelium

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SUMMARY

The olfactory epithelium (OE) of the mammal is uniquely suited as a model system for studying how neurogenesis and cell death interact to regulate neuron number during development and regeneration. To identify factors regulating neurogenesis and neuronal death in the OE, and to determine the mechanisms by which these factors act, investigators studied OE using two major experimental paradigms: tissue culture of OE; and ablation of the olfactory bulb or severing the olfactory nerve in adult animals, procedures that induce cell death and a subsequent surge of neurogenesis in the OE *in vivo*. These studies characterized the cellular stages in the olfactory receptor neuron (ORN) lineage, leading to the realization that at least three distinct stages of proliferating neuronal precursor cells are employed in generating ORNs. The identification of a number of factors that act to regulate proliferation and survival of ORNs and their precursors suggests that these multiple developmental stages may serve as control points at which cell number is regulated by extrinsic factors. *In vivo* surgical studies, which have shown that all cell types in the neuronal lineage of the OE undergo apoptotic cell death, support this idea. These studies, and the possible coregulation of neuronal birth and apoptosis in the OE, are discussed.

Keywords: **neurogenesis, apoptosis, programmed cell death, neuronal precursor cells, stem cells, transcription factors, *Mash1*, *Otx2*, growth factors, fibroblast growth factors, neurotrophins, transgenic mice.**

INTRODUCTION

The olfactory epithelium (OE) of the mammal is uniquely suited as a model system for studying how neurogenesis and apoptosis, or programmed cell death, interact to regulate neuron number during development and regeneration. Proliferation of neuronal precursor cells, differentiation of their progeny into olfactory receptor neurons (ORNs), and death and turnover of ORNs are processes that begin during embryonic development in the OE, and then continue throughout adult life (Graziadei and Monti-Graziadei, 1978, 1979). This capacity for continual nerve cell renewal led a number of different groups to perform studies that provide evidence suggesting that cell interactions can regulate neurogenesis and cell death in the OE *in vivo*. Death of cells in the OE can be upregulated in the adult mammal by lesioning the axons of ORNs or ablating their synaptic target tissue, the main olfactory bulb of the brain (Monti-Graziadei and Graziadei, 1979; Costanzo and Graziadei, 1983; Michel et al., 1994; Holcomb et al., 1995). This cell death in turn leads to increased mitotic activity in the neuronal precursor cells of the epithelium, which then produce new ORNs (Camara and Harding, 1984; Schwartz-Levey et al., 1991; Gordon et al., 1995).

The ability of the OE to renew its neuronal population throughout life raises the question of what properties of this tissue enable it, as opposed to most other regions of the mammalian nervous system, to regenerate neurons. For example, the fact

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that the OE of all vertebrates undergoes continuous neuron turnover and renewal throughout life would seem to guarantee the existence of a neuronal stem cell in this tissue. To try to determine if this is the case, and to understand the cellular and molecular basis of neuron renewal in the OE, we and others have established tissue culture systems to facilitate identification of the molecular factors involved (Noble et al., 1984; Schubert et al., 1985; Gonzales et al., 1985; Chuah et al., 1985, 1991; Coon et al., 1989; Calof and Chikaraishi, 1989; Pixley and Pun, 1990; Calof and Lander, 1991; Ronnett et al., 1991; Pixley, 1992; Mahanthappa and Schwarting, 1993; Calof et al., 1994a; Holcomb et al., 1995). This work led to the identification of a number of factors that can regulate proliferation and survival of OE cells, and in addition, to new insights concerning the cellular stages that lead from undifferentiated stem cell to mature ORNs.

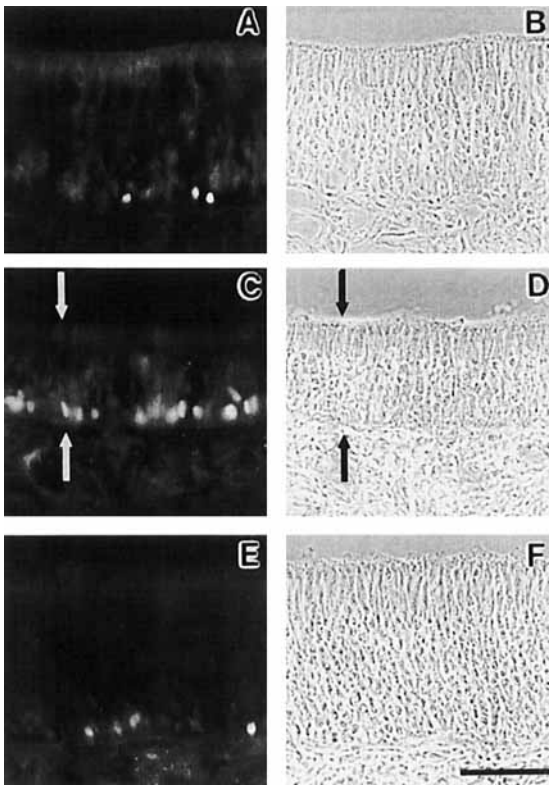
In this review we describe what is known concerning cellular stages in the neuronal lineage of the OE. Neuronal cell death in the OE following synaptic target tissue ablation *in vivo* is discussed, and the identification of factors that can regulate this process *in vitro,* and potentially *in vivo,* is also described. We outline information gained from *in vitro* studies on how ORNs are generated from their precursor cells, and what is known concerning regulation of this process by extrinsic factors. Finally, the relationship between ORN cell death and neurogenesis is discussed, along with possible bases by which these two processes may be coregulated.

CELLULAR STAGES IN THE ORN LINEAGE

Much of our detailed knowledge concerning cellular stages in the ORN lineage comes from tissue culture studies, using cell type specific markers to identify different cells of the OE and ^{3}H-thymidine and/or BrDU incorporation analysis to examine precursor–progeny relationships among cells. Several years ago, we established an explant culture system using embryonic day 14.5–15.5 mouse OE, in which three major cell types are distinguishable by antigenic markers, morphology, and differences in their migratory behavior (Calof and Chikaraishi, 1989; Calof and Lander, 1991). The three cell types are: *basal cells,* which express keratin intermediate filaments and do not migrate *in vitro,* instead remaining in tightly associated epithelial sheets within the main body of the explanted tissue; postmitotic *ORNs*, which express the neural cell adhesion molecule (NCAM, a neuron-specific marker in this system), and migrate away from OE explants and extend neurites when grown on appropriate extracellular matrix substrata (Calof and Lander, 1991; Calof et al., 1994b); and keratin-negative, NCAM-negative cells, which like ORNs are migratory, but do not have neurites and, unlike ORNs, incorporate ^{3}H-thymidine and divide in culture. In serum-free, defined conditions, these cells divide once and give rise to two daughter cells, which differentiate into ORNs and begin expressing NCAM within about 12 h of the terminal S phase; hence we call these cells the *immediate neuronal precursors* (*INPs*). INPs are the *in vitro* equivalents of the so-called "globose" basal cells of the OE, which have been shown to be the direct precursors of ORNs *in vivo* (cf. Graziadei and Monti-Graziadei, 1979; Mackay-Sim and Kittel, 1991; Schwartz-Levey et al., 1991; Caggiano et al., 1994). We demonstrated that INPs behave as committed neuronal precursor cells, capable of undergoing a limited number of amplification divisions in response to appropriate exogenous factors (see below), and as such fit the description of neuronal "transit amplifying cells" in the ORN lineage (DeHamer et al., 1994; Hall and Watt, 1989; Potten and Loeffler, 1990). Evidence for the existence of a potential neuronal stem cell, which gives rise to INPs in explant cultures, was also recently obtained, although no marker specific for this cell has yet been identified (DeHamer et al., 1994; see below).

More recently, we performed studies to try to determine the true complexity of the ORN lineage. How many precursor cell stages lie between the self-renewing stem cells of the OE and the INPs that are committed to giving rise to ORNs? We gained insight into this question by studying the dynamics of expression of several different developmentally regulated transcription factors during OE neurogenesis *in vivo* and *in vitro.* Two of these, *Mash1*, a mammalian homologue of the *Drosophila achaete-scute* proneural genes (Johnson et al., 1990), and *Otx2,* a murine homeogene related to the *Drosophila* gene *orthodenticle* (Simeone et al., 1992), have so far proved interesting.

Our work on *Mash1* expression indicates that expression of this transcription factor demarcates a distinct stage of neuronal precursor in the ORN lineage, but that MASH1$^+$ cells are not the true stem cells of the OE. Our choice of MASH1 as a



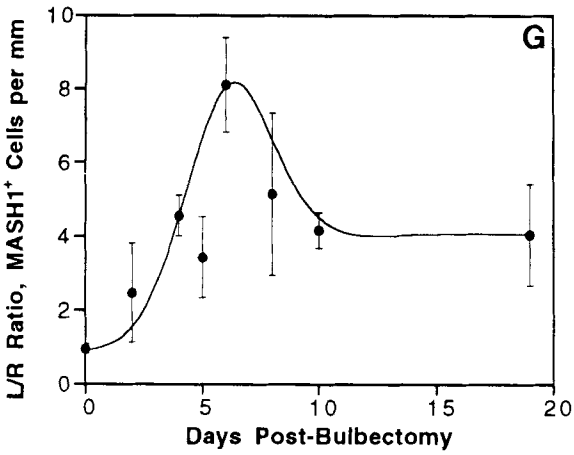


Figure 1 MASH1 expressing cells increase in number when neurogenesis is stimulated *in vivo*. Adult male mice were subjected to unilateral (left) bulbectomy and sacrificed at various times from 2–19 days postsurgery. Several unoperated control animals were also sacrificed. Cryostat sections (12 μm) through the OE were taken in the horizontal plane (so that both left and right OE were present on each section), processed for MASH1 immunoreactivity as described (Gordon et al., 1995), and examined by fluorescence and phase contrast microscopy. (A–F) Photos of the OE (A,B) of an unoperated animal are compared with photos of (C,D) the left (ipsilateral, operated side) and (E,F) the right (contralateral, control side) septal OE from a single section of an animal sacrificed 5 days after bulbectomy. (A,C,E) MASH1 immunofluorescence is shown; (B,D,F) corresponding phase contrast images. The photos show a marked increase in the number of MASH1$^+$ cells (C) on the ipsilateral side of the operated animal, compared to (E) the contralateral or (A) unoperated controls. (C,D) Also apparent is the decrease in overall thickness of the OE (the result of cell loss) on the ipsilateral side of the operated animal (arrows), when compared to either of the controls. Bar-50 μm. (G) Stained sections such as those shown in (A–F) were used to determine the number of MASH1$^+$ cells per linear distance along the OE on both sides of animals sacrificed at various times following unilateral bulbectomy. To normalize for interanimal variability in the number of MASH1$^+$ cells, data were converted to a ratio of MASH1$^+$ cells/mm on the operated side to MASH1$^+$ cells/mm on the unoperated side, calculated for each animal. The data are plotted as a function of time following bulbectomy. Each data point represents the average of results obtained from at least three animals ± S.E.M. For each animal, the data were acquired from viewing the OE lying along the posterior part of the nasal septum, in multiple sections, covering several millimeter of OE. A smooth, freehand curve was drawn through the data points. (From Gordon et al., *Mol. Cell. Neurosci.* **6**:363–379, © 1995 Academic Press, reprinted with permission.)

potential marker for ORN precursors was initially motivated by two findings from other labs. First, MASH1 is expressed in regions of the embryonic murine nervous system where proliferating neuronal precursors are present (Lo et al., 1991). Second, and even more significantly, disruption of the *Mash1* gene by homologous recombination in mice results in a profound reduction in the numbers of ORNs that are generated during development (neuronal lineages in the autonomic and enteric nervous systems are also affected; Guillemot et al., 1993). Using an antibody to MASH1, we examined MASH1 expression in embryonic OE cultures, in normal adult mice, and in adult mice at various times following olfactory bulbectomy (surgical removal of the olfactory bulb, which results in the death of ORNs and a subsequent surge in neurogenesis in the OE) (Gordon et al., 1995). Our results indicate that MASH1 is expressed in a population of cells morphologically similar to INPs (*in vitro*) or globose basal cells (*in vivo*), but several-fold less abundant. When neurogenesis is transiently induced in the OE *in vivo* following bulbectomy, a transient surge in the number of MASH1

expressing cells in the OE ensues (Fig. 1). This property of MASH1$^+$ cells, taken together with their high ^{3}H-thymidine labeling index *in vitro* and *in vivo* (e.g., in adult, unstimulated OE, ~40% of MASH1$^+$ cells are labeled with a 2-h pulse of ^{3}H-thymidine administered immediately prior to sacrifice), indicate that MASH1$^+$ cells behave, like INPs, as transit amplifying cells in the OE. The expansion of MASH1 cell numbers in response to mitogenic stimulation (bulbectomy) is characteristic of the symmetric amplifying divisions of transit amplifying cells, rather than the asymmetric self-renewing divisions of stem cells (Hall and Watt, 1989; Potten and Loeffler, 1990); and the high ^{3}H-thymidine labeling index of MASH1$^+$ cells would be highly unusual in stem cells, which typically cannot be labeled with brief pulses of S-phase markers due to their extremely long cell cycle times (e.g. Cotsarelis et al., 1990; Jones et al., 1995).

In addition, our studies examined the numbers and proliferative states of MASH1-expressing cells under several different conditions: as neurogenesis winds down *in vitro* (in normal explant cultures); when neurogenesis is sustained for long periods in explant cultures; and when neurogenesis is transiently increased *in vivo* following bulbectomy. In all of these situations, the results were indicative of a precursor–product relationship between MASH1$^+$ cells and INPs (Gordon et al., 1995). This is illustrated in Figure 2 showing the results of experiments in which unilaterally bulbectomized mice and unoperated control animals were given pulses of ^{3}H-thymidine 2 h prior to sacrifice at a number of different postbulbectomy time points. The OE was then processed for MASH1 immunoreactivity and autoradiography, and the numbers of cells in the basal half of the OE that were ^{3}H-thymidine$^+$, MASH1$^+$, and positive for both markers were counted. The data show an early burst of proliferation in the MASH1$^+$ cell population (solid line), which then declines as MASH1$^+$ cells give rise to MASH1$^-$ INPs that then amplify their own numbers (dashed line) prior to giving rise to neurons. Altogether, the data suggest that *Mash1* expression demarcates a neuronal transit amplifying cell, which lies immediately upstream of the INP, but downstream of the stem cell, in the ORN lineage. Furthermore, our results suggest that the crucial role of *Mash1* in olfactory neurogenesis (Guillemot et al., 1993) is due to expression of MASH1 by cells within the ORN lineage itself.

Mash1 has a very restricted pattern of expression, both in the OE and elsewhere in the develop-

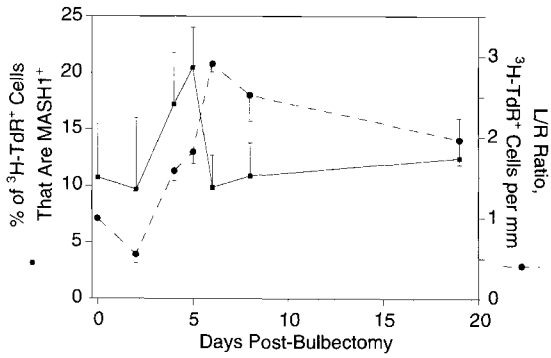


Figure 2 MASH1$^+$ cells proliferate prior to INPs when neurogenesis is induced *in vivo*. Adult male mice were subjected to unilateral (left) bulbectomy and sacrificed at various times from 2–19 days postsurgery. Unoperated control animals were also sacrificed. In all cases, animals were given two sequential injections of ^{3}H-TdR (each 20 μCi/g body weight; specific activity was 70–90 Ci/mmol) at 2 and 1 h prior to sacrifice, in order to label cells in the S phase with high sensitivity. Cryostat sections (12 μm) were taken and processed as in Figure 1, except that sections were additionally processed for autoradiography as described (Gordon et al., 1995). Data were obtained from counting the number of cells/mm of OE that were in the basal half of the epithelium and were MASH1$^+$, had incorporated ^{3}H-TdR, or were both MASH1$^+$ and ^{3}H-TdR$^+$. For each animal, data were collected from several millimeters of septal OE in each of multiple sections, and at least three animals were examined for each time point. The data are presented in two ways. The dashed line shows the number of ^{3}H-TdR$^+$ cells/mm in the OE on the operated (left) side, normalized to the number of ^{3}H-TdR$^+$ cells/mm on the unoperated (right) side. This provides a measure of overall proliferation in the OE on the bulbectomized side. The solid line shows, for the bulbectomized side of each animal, the percent of ^{3}H-TdR$^+$ cells that are also MASH1$^+$ (calculated for each section examined and averaged over the total number of sections). This provides a measure of the relative contribution of MASH1$^+$ cells to overall proliferation. In both cases, the data points represent mean ± S.E.M. The data indicate that, at early times following bulbectomy, MASH1$^+$ cells contribute preferentially to the increase in overall proliferation, whereas by the time overall proliferation peaks, MASH1$^+$ cells no longer contribute preferentially. [Note that the value for the percent of ^{3}H-TdR$^+$ cells that are also MASH1$^+$ at 5 days is significantly different from the values at either 0 days ($p = 0.006$) or 6 days ($p = 0.001$), whereas the values at 0 and 6 days are not significantly different from each other]. Thus, in response to bulbectomy, increased proliferation of MASH1$^+$ cells precedes increased proliferation of cells in the basal half of the OE as a whole (80–90% of which are MASH1$^-$). (From Gordon et al., *Mol. Cell. Neurosci.* **6**: 363–379, © 1995 Academic Press, reprinted with permission.)

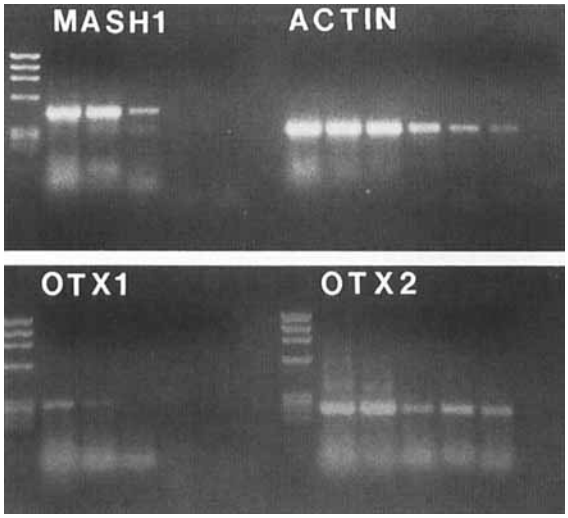


Figure 3 RT-PCR analysis of *Mash1, Otx1, Otx2,* and actin mRNA levels in E14.5–15.5 OE. Total RNA was isolated from E14.5–15.5 nasal turbinates using Ultraspec RNA isolation mixture (Biotex). First strand cDNA was synthesized from 10 μg of total RNA in 20 μL of reaction mixture containing 1× RT buffer, 1 m*M* dNTPs, 100 pmol random hexamer, and 10 U of AMV RT (Promega) for 1 h at 37°C. Desired amounts of cDNA mix were added to a PCR mix containing 1× PCR buffer, 1 m*M* $MgCl_2$, 250 μM dNTPs, 0.1 mg/mL BSA, 0.5 μM each of forward and reverse primers, and 2.5 U of Taq DNA polymerase (Gibco-BRL) to a final volume of 20 μL. The cycling parameters were denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min. Cycle numbers used were 40 cycles for *Mash1, Otx1, Otx2*; 30 cycles for actin. After reverse transcription with random primers, separate PCR reactions were performed for each set of specific primers. Specific primers were:

Mash1: 5′-CTC TTA GCC CAG AGG AAC-3′ (forward)
5′-GGT GAA GGA CAC TTG CAC-3′ (reverse)
Otx1: 5′-CGT ATC TAG CTC TGC TTC-3′ (forward)
5′-CCT GGC CAT AGG ACA TAG-3′ (reverse)
Otx2: 5′-CTC TAG TAC CTC AGT CCC A-3′ (forward)
5′-GTC CAG GAA GCT GGT GAT G-3′ (reverse)
B actin: 5′-TCA TGA AGT GTG ACG TTG ACA TCC-3′ (forward)
5′-GTA AAA CGC AGC TCA GTA ACA GTC-3′ (reverse)

A series of different cDNA amounts was used for PCR, and amplified products were separated on 2% agarose gels. In the photographs of *Otx1, Otx2,* and *Mash1* gels, lanes are, from left, a DNA size marker ($\phi \times 174$), PCR products from 4, 2, 1, 0.5, and 0.25 μL of cDNA input. For actin, lanes are, from left, a DNA size marker, PCR products from 2, 1, 0.5, 0.25, 0.125, and 0.0625 μL cDNA input. Expected sizes of amplified fragments are: *Otx1,* 290 bp; *Otx2,* 242 bp; *Mash1,* 454 bp; actin, 290 bp. No-RT controls gave no amplification products in the subsequent PCR reactions (not shown). The sizes of the DNA marker fragments ($\phi \times 174$) are from top: 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, and 271 bp.

ing nervous system. In contrast, expression of *Otx2* appears to be much more widespread among neural precursor cells within the anterior regions of the developing nervous system, including the OE, during periods when neurons are actively generated (e.g., E12.5; Simeone et al., 1993). To determine if *Otx2* might be a useful marker for ORN precursors, we first performed an analysis of levels of expression of *Mash1*, *Otx2,* and *Otx1,* another murine *orthodenticle* homologue (Simeone et al., 1992), using RNA made from nasal turbinates isolated from E14.5–15.5 mouse embryos, the same age and tissue that serves as a source of cells for our OE explant cultures. This analysis, illustrated in Figure 3, shows that *Otx2* and *Mash1* messages are both relatively abundant, while *Otx1* appears to be the least abundant message of the three tested. *In situ* hybridization for *Otx2* mRNA, shown in Figure 4, indicates that *Otx2* is expressed by a significant proportion of migratory cells (47.1 + 2.1% S.E.M.) in 12-h OE explant cultures. Our previous studies showed that the migratory cells in these cultures consist entirely of ORNs and ORN precursors (Calof and Chikaraishi, 1989; Calof and Lander, 1991). However, essentially no *Otx2* expressing cells have neurites (1 out of 327 migratory cells counted in 10 random fields), suggesting that *Otx2* is not expressed by differentiated ORNs. Although confirmation of *Otx2* as a precursor-specific marker awaits ^{3}H-thymidine incorporation analysis and double-labeling experiments using other OE cell type specific markers (e.g., anti-MASH1), both the number and morphology of *Otx2*-expressing cells suggest that *Otx2* may be a global marker for ORN progenitors, a possibility that we are currently investigating.

The theme that is emerging from these studies is the complexity of this neuronal lineage: there appear to be at least two distinct stages of proliferating neuronal precursor cells interposed between the

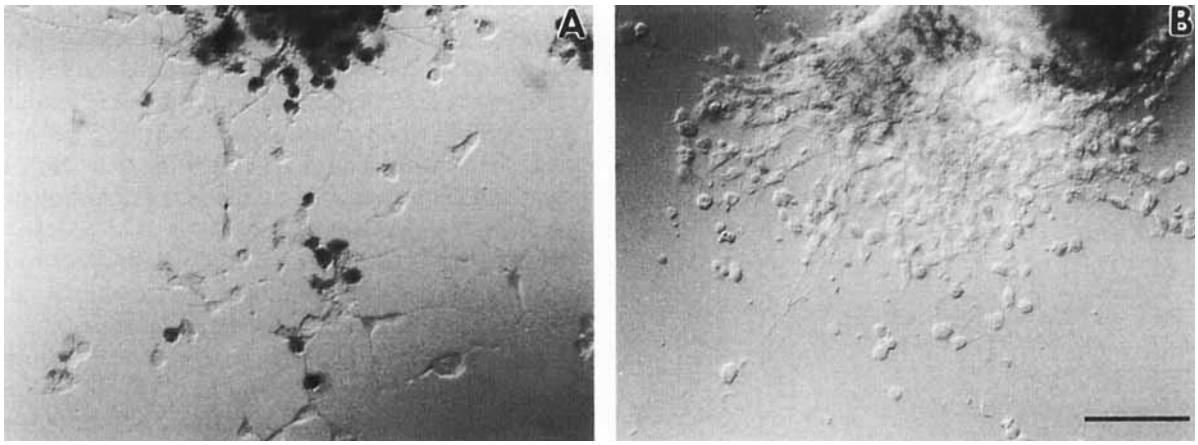


Figure 4 *In situ* hybridization for *Otx2* mRNA in OE explant cultures. E14.5 OE explants were cultured in serum-free medium on polylysine- and merosin-coated coverslips as described (DeHamer et al., 1994) for 12 h and *in situ* hybridization with dioxygenin (DIG)-labeled *Otx2* (A) antisense or (B) sense probes was performed. DIG-labeled *Otx2* antisense and sense probes (~250 bp) were synthesized from pMOTX2 (gift of Dr. A. Simeone through Dr. S. McConnell). Hybridization was performed at 60°C in 10× Denhardt's, 50% formamide, 4× SSC, and 250 μg/mL yeast tRNA containing 300 ng/mL of antisense or sense probe. Unbound probe was removed by wash at 55°C in 50% formamide and 5× SSC, RNase treatment (20 μg/mL) at 37°C, again washed in 50% formamide and 2× SSC, finally at room temperature in 2× SSC. The remaining probe was reacted with anti-DIG antibody and color was developed in NBT/BCIP by following the manufacturer's recommendation (BMB, DIG nucleic acid detection kit) with modification described by Schaeren-Wiemers and Gerfin-Moser (1993). Note that *Otx2* positive cells are nonprocess bearing cells. Bar = 50 μm.

postmitotic ORN and the stem cell that is ultimately responsible for the ability of the OE to continually generate neurons. The role of these multiple developmental stages is not known. However, studies discussed below suggest that each cellular stage in the ORN lineage serves as a point at which extrinsic factors can act to control cell number and, perhaps, cell fate.

APOPTOSIS IN THE NEURONAL LINEAGE OF THE OE

Previous work from a number of different laboratories demonstrated that when one olfactory bulb is removed from an adult animal (unilateral bulbectomy), nearly all ORNs in the ipsilateral OE die (e.g., Costanzo and Graziadei, 1983). The OE then decreases in thickness as cells degenerate. Despite the fact that cells in the basal compartment of the OE then proliferate and many new ORNs are generated (Schwartz-Levey et al., 1991; Gordon et al., 1995), in the absence of its synaptic target tissue the OE never reaches its original thickness. This appears to result from newly generated ORNs being able to survive for only a short time when the olfactory bulb is absent (Schwob et al., 1992; Carr and Farbman, 1992, 1993). In addition, it was reported that the rate of generation of new ORNs is permanently elevated following bulbectomy, suggesting that this ongoing ORN death might somehow play a role in regulating proliferation of neuronal precursors in the OE (Carr and Farbman, 1992).

Until recently, it was not known whether the death that cells undergo in the OE following bulbectomy is apoptotic cell death, or even what cell types are induced to die. Although it had been reported that DNA fragmentation occurs in the OE when the olfactory bulb is removed, this work was done using agarose gel electrophoresis to evaluate fragmentation, and therefore could not provide information about the numbers or types of cells induced to die (Michel et al., 1994). To approach these questions, we performed an analysis of cell death in the OE under three conditions: in normal adult mice; in adult mice subjected to unilateral olfactory bulbectomy; and in primary cell cultures

derived from embryonic mouse OE. To quantify apoptotic cell death, we used the TUNEL technique (DNA end-labeling with deoxynucleotide terminal transferase and dUTP-biotin) to test for DNA fragmentation in cells *in vivo* and *in vitro* (Arends and Wyllie, 1991; Gavrieli et al., 1992; Deckwerth and Johnson, 1993). We combined this technique with the use of cell-type specific antibody markers and ^{3}H-thymidine incorporation to determine which cells in the ORN lineage die, and with what time course, when they are deprived of synaptic contact with the olfactory bulb.

In Figure 5, an example of an *in vivo* experiment is shown. In this experiment, TUNEL labeling was combined with anti-NCAM (neural cell adhesion molecule) immunohistochemistry (Calof and Chikaraishi, 1989) so that the number of NCAM$^+$ ORNs induced to die 24 h following bulbectomy could be quantified. Figure 5(A–C) shows septal OE on the side ipsilateral to the surgery; (D,E) show the septal OE immediately opposite, on the contralateral, unoperated side. In (A), the TUNEL staining (white dots; e.g., arrow) shows the nuclei of cells with fragmented DNA; many cells are clearly undergoing DNA fragmentation, and therefore apoptotic cell death, by 24 h postsurgery. The double labeling with anti-NCAM in Figure 5(B) shows that the great majority of apoptotic cells are ORNs. As shown in Figure 5(D), there was virtually no TUNEL staining in the contralateral, unoperated OE. It is also apparent from these photographs that a high level of TUNEL staining is evident before overt signs of morphological degeneration are observed in the OE [compare Fig. 5(C) with (F)], indicating that the onset of extensive cell death in the OE occurs several days earlier than estimates previously indicated, based on measurements of OE thickness alone.

We assessed the time course and extent of apoptosis in the OE following bulbectomy in animals sacrificed from 12 h to 84 days after surgery. Data from these experiments are summarized in Figure 6. In OE on the bulbectomized side, the number of TUNEL$^+$ cells increases sharply by 12 h postsurgery and is maximal at 2 days. The number of TUNEL$^+$ cells then declines rapidly to near-normal levels but still remains elevated over that seen in the contralateral OE at all time points tested. The mean thickness of OE on the operated side is also shown (cf. Costanzo and Graziadei, 1983; Schwartz-Levey et al., 1991). When we combined TUNEL staining with immunohistochemistry and ^{3}H-thymidine incorporation analysis to identify the types of cells undergoing apoptotic death, we found that cells at all stages in the OE neuronal lineage (proliferating neuronal precursor cells, immature ORNs, and mature ORNs) undergo apoptosis in the acutely bulbectomized animal (24 h following bulbectomy). Bulbectomy does not induce apoptosis of two other cell types whose role, if any, in the ORN lineage is uncertain; keratin-expressing horizontal basal cells, and supporting or sustentacular cells (Holcomb et al., 1995). In the chronically bulbectomized OE, the increase in apoptosis that is observed is accounted for entirely by an increase in the death of mature ORNs, suggesting that the factors that mediate the survival of mature ORNs may differ from those mediating the survival of immature ORNs and neuronal precursors (Holcomb et al., 1995). Thus, apoptosis appears to regulate neuronal number in the OE at multiple stages in the neuronal lineage, an observation that has been made for other neuronal lineages as well (e.g., Birren and Anderson, 1993; DiCicco-Bloom et al., 1993; Verdi and Anderson, 1994).

Our studies *in vitro* confirmed that embryonic ORNs and their precursors also undergo cell death when explanted into culture. Like the cell death that occurs following ablation of the olfactory bulb *in vivo,* pharmacological experiments and TUNEL staining demonstrate that olfactory neuronal cell death *in vitro* has the characteristics of apoptosis. Interestingly, the onset of apoptosis shows a similar time course *in vivo,* following bulbectomy, and *in vitro,* following explanation of dissociated olfactory neuronal cells into culture (Fig. 7).

To begin to identify factors that might mediate olfactory neuronal cell survival *in vivo,* we used *in vitro* assays to test agents that prevent apoptosis in other cells, including aurintricarboxylic acid (ATA), a membrane-permeant analog of cyclic AMP (CPT-cAMP), and members of the neurotrophin family of polypeptide growth factors (Holcomb et al., 1995). [Neurotrophins are known to be expressed in the olfactory bulb, making them good candidates for potential target-derived trophic factors in this system (e.g., Large et al., 1986; Maisonpierre et al., 1990; Guthrie and Gall, 1991).] ATA and CPT-cAMP are each able to promote survival of a fraction of cultured ORNs, as are three neurotrophins—brain derived neurotrophic factor, neurotrophin-3, and neurotrophin-5, but not nerve growth factor (Holcomb et al., 1995). We used immunohistochemical methods to determine if the neurotrophin tyrosine kinase re-

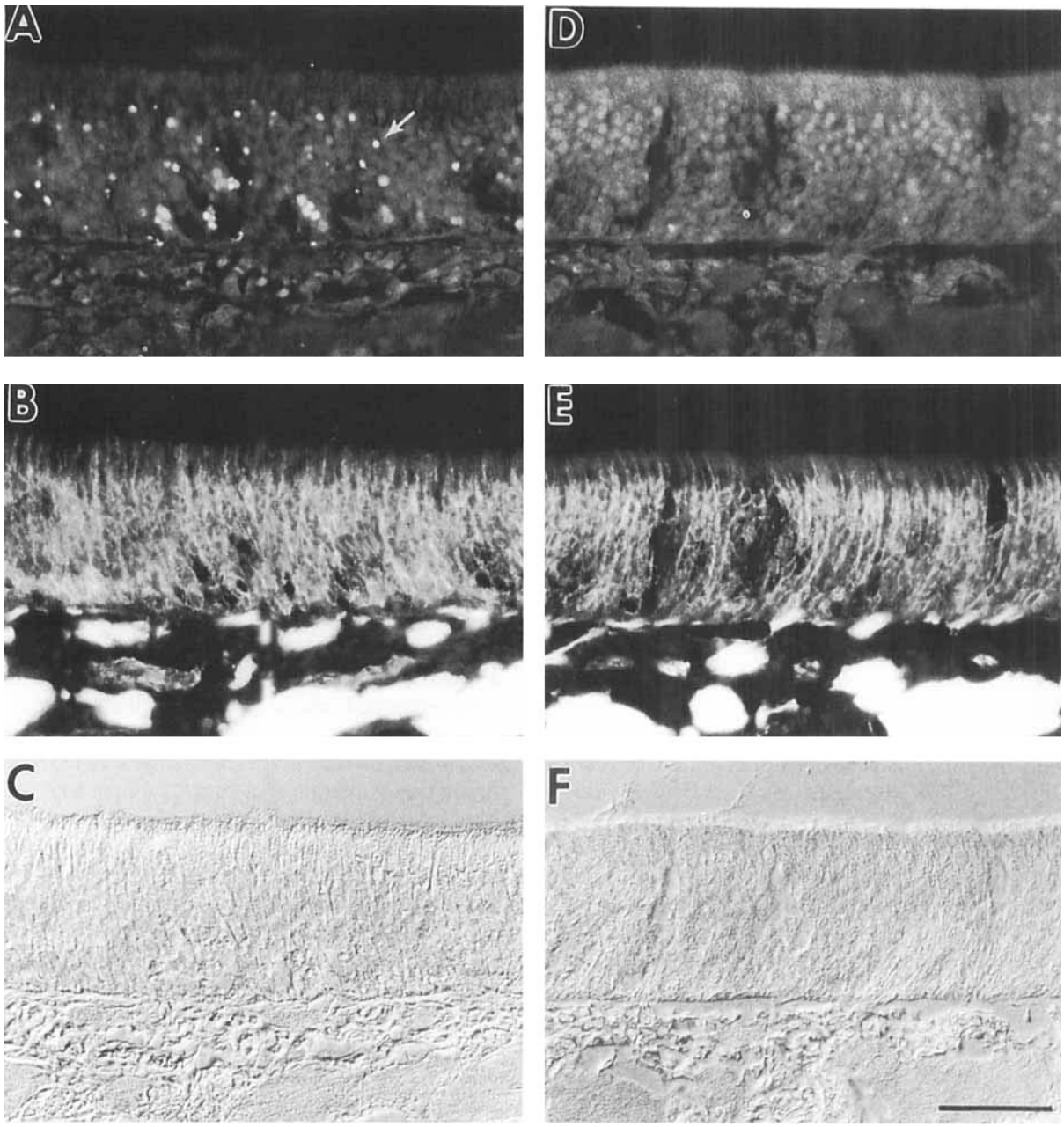


Figure 5 DNA fragmentation in the olfactory epithelium following unilateral olfactory bulbectomy. Adult male mice were anesthetized and a small suction tube was used to selectively remove the left olfactory bulb without causing injury to the contralateral olfactory bulb or to the brain. At time points ranging from 12 h to 84 days following surgery, animals were sacrificed and the region of the nose containing the OE was dissected and fixed by freeze substitution as described (Holcomb et al., 1995). After decalcification for 7 days in ~390 m*M* EDTA, pH 7.1, the tissue was sectioned in the horizontal plane in 12-μm sections with a cryostat. Sections of OE were stained for DNA fragmentation using deoxynucleotide terminal transferase end labeling of DNA fragments with biotinylated dUTP and a fluorescent avidin, a modification of the TUNEL technique of Gavrieli et al. (1992). (A–C) Photos show the bulbectomized OE and (D–F) contralateral OE immediately opposite from an animal sacrificed at 24 h postbulbectomy. (A,D) Fluorescein optics showing TUNEL staining; arrow in (A) indicates TUNEL$^+$ cell. (B,E) Rhodamine optics showing NCAM immunoreactivity in the same sections. (C,F) Nomarski optics. Bar = 50 μm. (From Calof et al., *Growth Factors as Drugs for Neurological and Sensory Disorders*, © 1995 The Ciba Foundation, reprinted with permission.)

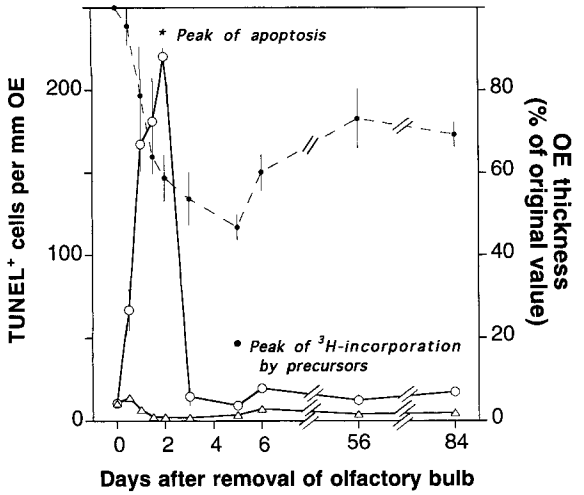


Figure 6 Time course of DNA fragmentation following unilateral bulbectomy. Cryostat sections of OE from unoperated (control) and bulbectomized mice (sacrificed at postoperative time points indicated) were processed for TUNEL as described (Holcomb et al., 1995). TUNEL$^+$ cells were counted in sections of septal OE on the bulbectomized side (open circles), contralateral side (open triangles), and from unoperated animals (time = 0). Mean numbers of TUNEL$^+$ cells/mm OE (± S.E.M.) are plotted, together with changes in the average thickness of the bulbectomized OE (solid circles), over time following bulbectomy. Where error bars are not seen, the error was small enough to be obscured by the symbol representing the data point. Differences between bulbectomized and contralateral OE were statistically significant for all times ≤ 2 and ≥ 6 days ($p \leq 0.02$ except at 56 days, where $p = 0.055$; Student's t test; Glantz, 1992). (From Holcomb et al., *Dev. Biol.* **172**: 307–323, © 1995 Academic Press, reprinted with permission.)

ceptors trkB and trkC are expressed in OE, and, consistent with the effects we see on ORN survival *in vitro,* we found that these two receptors are expressed by fractions of ORNs scattered throughout neonatal OE (Holcomb et al., 1995; cf. Deckner et al., 1993). However, we do not think it likely that neurotrophins are the only growth factors that mediate survival of ORNs: the survival-promoting effects of individual neurotrophins *in vitro* are never as large as those seen with ATA or CPT-cAMP; and when we have tested combinations of different neurotrophins, we did not observe an increase in cell survival (Holcomb et al., 1995; J.D.H. and A.L.C., unpub. observ.). The most plausible explanation for this is that other factors, in addition to neurotrophins, are necessary for complete ORN survival (see also Mahanthappa and Schwarting, 1993).

In light of our finding that OE neuronal precursors undergo apoptosis, we recently began to analyze cell death in the OE of *Mash1*$^{-/-}$ embryos. We found that few ORNs ever appear in *Mash1*$^{-/-}$ OE, indicating that the lack of ORNs at birth reflects a defect in neuronal production (as opposed to neuronal survival). Interestingly, we observe an elevated level of cell death in the NCAM$^-$ cells in the E14–15 *Mash1*$^{-/-}$ OE (compared to wild-type and heterozygote controls) as revealed by TUNEL staining (Calof et al., 1995; J.S. and A.L.C., data not shown). Because the majority of the NCAM$^-$ cells in the epithelium at this age (E14–15) are neuronal precursors

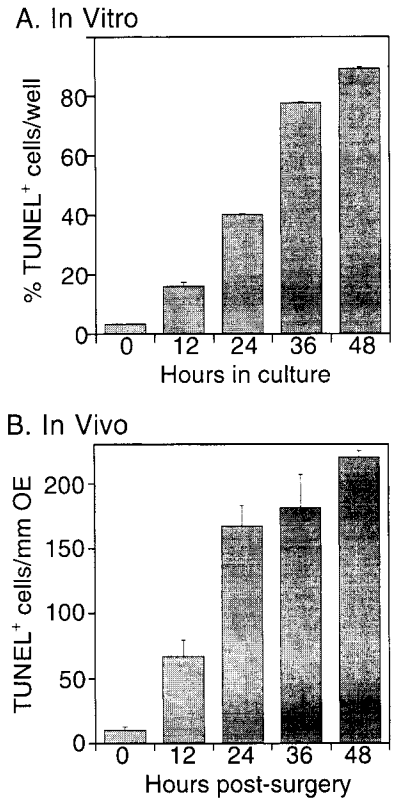


Figure 7 DNA fragmentation in target-deprived olfactory neuronal cells occurs over similar time courses *in vitro* and *in vivo*. (A) Dissociated olfactory neuronal cells from E16.5–17.5 CD-1 embryos were plated at a density of ~3×10^3 cells/well in 96-well tissue culture plates as described (Holcomb et al., 1995). Cells were fixed at indicated times and stained for TUNEL. For the $t = 0$ time point, cells were incubated for 30 min at 37°C prior to fixation. The percentage of total cells per well that are TUNEL$^+$ is plotted for each time point. Data show mean ± S.E.M. of triplicate wells. (B) Data regraphed from Figure 6.

(Calof and Chikaraishi, 1989), we hypothesize that, in *Mash1*$^{-/-}$ animals, neuronal precursor cells are produced, but most undergo apoptosis without generating ORNs. A role for *Mash1* in regulating expression of genes that mediate survival of neuronal precursor cells in the ORN lineage is a possibility that we are currently investigating.

REGULATION OF NEUROGENESIS IN THE OE

Neurogenesis ceases after about 24 h in OE explants cultured in serum-free, defined medium in the absence of exogenous growth factors (Calof and Chikaraishi, 1989). In contrast, neurogenesis *in vivo* occurs continually throughout the lifetime of the organism. This abrupt termination of neurogenesis *in vitro* was used by our laboratory as the basis for a screen to identify polypeptide growth factors capable of promoting prolonged neurogenesis in OE explant cultures. Our findings indicate that members of the fibroblast growth factor (FGF) family are able to promote prolonged neurogenesis, and further that FGFs act in two ways (DeHamer et al., 1994). First, FGFs act to increase the likelihood that INPs divide twice, rather than once, before generating ORNs. This action requires exposure of INPs to FGFs by early G_1 of their cell cycle, the phase at which their commitment to terminal differentiation would be expected to occur (Soprano and Cosenza, 1992). This is similar to the way in which FGFs are believed to act in myogenesis, where FGFs act to promote myoblast proliferation by repressing terminal differentiation in G_1, thereby allowing cells to progress through additional cell cycles (Clegg et al., 1987). Further, observation of the action of FGFs in promoting divisions of INPs while leaving their neuronal fate unaffected (INPs quantitatively give rise to ORNs even when cultured in FGFs; neuronal differentiation is simply delayed by the additional cell cycles) is responsible for our notion of INPs as neuronal transit amplifying cells (DeHamer et al., 1994).

The second action of FGFs is to cause a distinct subpopulation of OE explants to continually generate large numbers of neurons for at least several days. Only 5–8% of explants continue to generate large numbers of ORNs at late times in culture, suggesting that an early, rare progenitor, possibly a neuronal stem cell, is present in these explants, and that FGF supports the proliferation and/or survival of this cell (DeHamer et al., 1994). Our estimates of the abundance of this cell put it at a frequency of ~1/2500 INPs, suggesting that these cells may lie far "upstream" of INPs in the ORN lineage (J.S.M., J.S., and A.L.C., unpub. observ.). Our current hypothesis is that this cell may be the neuronal stem cell that is ultimately responsible for the ability of the OE to continually generate neurons.

Stimulation of ORN production in OE cultures was reported by two other groups. Coculture of neonatal rat OE with astrocytes was shown to prolong the generation of ORNs (Pixley, 1992), an effect that may be attributable to production of FGFs by these cells (Woodward et al., 1992; Baird, 1994). Mahanthappa and Schwarting (1993) reported that TGF-β2 stimulates neurogenesis in postnatal rat OE cultures, an effect that we were not able to reproduce in our assay system (DeHamer et al., 1994). However, the study by Mahanthappa and Schwarting (1993) left open the possibility that the role of TGF-β2 is to serve as a survival factor for newly generated ORNs, rather than as a proliferation factor for neuronal precursors. In addition, differences in the age of tissue assayed, and/or complications arising from endogenous production of TGF-β2 by OE cells differentially present in the two culture systems (cf. Millan et al., 1991), may have contributed to the different results obtained in the two studies.

Recently, we developed an approach to obtain more direct evidence for the existence of early progenitors (potential stem cells) in the OE and to learn more about growth conditions for these cells. To isolate early progenitors, a neuronal cell fraction containing purified INPs and ORNs is separated from the basal cells in E14–15 OE suspension cultures on the basis of differential cell adhesion (Calof and Lander, 1991; Calof et al., 1996); then dishes coated with antibodies to NCAM (a specific marker for differentiated ORNs) are used to remove ORNs (Hagiwara et al., 1995; J. S. Mumm, J. Shou, and A. L. Calof, submitted). When the resulting purified INP fraction is cultured, most cells quickly give rise to neurons. However, if the cells are plated on top of stromal fibroblasts from the embryonic OE, small numbers of colonies of undifferentiated cells are visible after 7 days in culture [Fig. 8(A)]. [Typically, the INP fraction is isolated from OE purified from the Rosa 26 transgenic mouse strain (Friedrich and Soriano, 1991), which expresses *lacZ* in all cells, while the stromal cells are isolated from wild-type mice; colonies are then

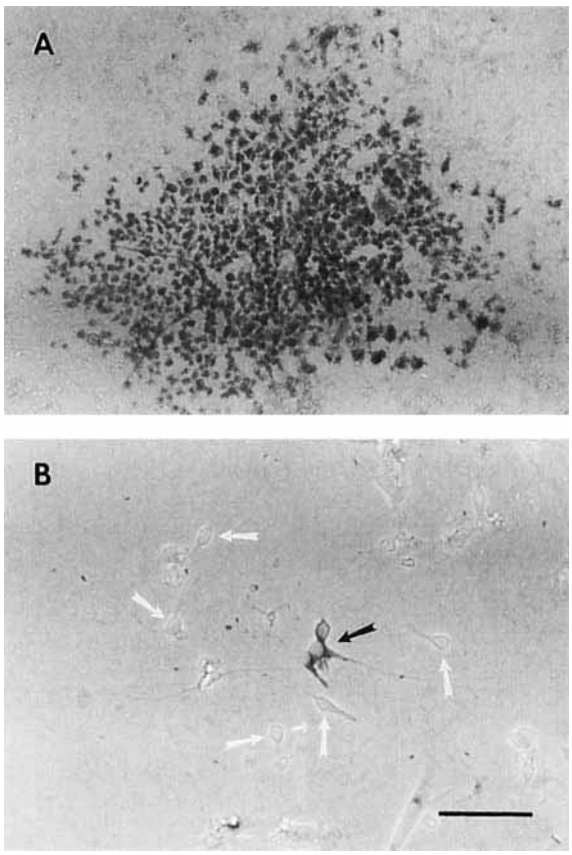


Figure 8 Purified ORN progenitors form colonies and continue to generate neurons for 7 days in culture. (A) NCAM progenitor cells were purified from the dissociated neuronal cell fraction of E14.5–15.5 Rosa26 transgenic mice, as described in the text. After coculture over a monolayer of mitomycin-treated (10 μg/mL for 2 h) stromal fibroblasts for 7 days, cultures were fixed (15 min in 0.5% glutaraldehyde, 2 m*M* $MgCl_2$, 5% sucrose in PBS, pH 7.5), permeablized with 0.1% Triton, 0.01% deoxycholic acid, and 2 m*M* $MgCl_2$ in PBS (pH 7.5) for 1 h, and then placed in X-Gal staining solution [0.1% Triton, 0.01% deoxycholic acid, 2 m*M* $MgCl_2$, 5 m*M* $K_3Fe(CN)_6$, and 5 m*M* $K_4Fe(CN)_6$ in PBS (pH 7.5), plus 320 mg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] for 24 h at 37°C. Colonies of cells arise at the rate of approximately 1/800 panned, purified progenitor cells initially plated. The majority of these colonies contain cells with morphological characteristics of undifferentiated progenitor cells: they are small, round, and do not bear neurites. (B) Progenitor cell colonies were first cultured for 7 days on stromal fibroblasts, then dissociated from feeder cell layers and replated over E14.5 CD-1 derived OE explants that had been precultured for 24 h. Cells with morphological characteristics of ORNs can be seen by X-Gal staining. The black arrow shows a small clump of 3 *lacZ*-positive cells, indicating that these cells were derived from replated Rosa26 progenitors. White arrows indicate the *lacZ*-negative ORNs from the CD-1 explants, for morphological comparison. Bar = 50 μm. Micrograph in (B) is approximately 4× the magnification of micrograph shown in (A).

visualized using β-galactosidase histochemistry (Xgal staining).] When these colonies are dissociated from their stromal feeder layers, and replated under conditions that promote neuronal differentiation, cells that are morphologically identical to ORNs are formed [Fig. 8(B)]. Staining with antibodies to NCAM demonstrates that these β-galactosidase-expressing cells are in fact ORNs, and ^{3}H-thymidine incorporation analysis indicates that the precursors of these ORNs are still dividing after 7 days in coculture with stromal fibroblasts (J. S. Mumm, J. Shou, and A. L. Calof, submitted). These results suggest very early progenitors in the ORN lineage can be isolated and that factors produced by stromal cells (possibly including FGFs; cf. Mason et al., 1994) can maintain these cells and their capacity to generate neurons for at least 7 days in culture.

ARE NEURONAL BIRTH AND DEATH COREGULATED IN THE OE?

Proliferation of neuronal precursors in the OE (detected as ^{3}H-TdR incorporation by cells in the basal compartment of the epithelium) increases following bulbectomy, reaching a peak 5–6 days postsurgery in the mouse (Fig. 6; cf. Schwartz–Levey et al., 1991). Cell loss in the OE (measured as epithelial thickness) follows a similar time course: epithelial thickness reaches its minimum at about 5 days postbulbectomy, then increases again to approximately 70% of its original value (Fig. 6; Costanzo and Graziadei, 1983; Schwartz–Levey et al., 1991). This temporal correlation between neuronal cell loss in the OE and ^{3}H-TdR incorporation by ORN precursors suggests a possible causal relationship between the two events: differentiated ORNs might somehow provide a signal that feeds back to inhibit proliferation of their own precursors. Such a regulatory mechanism was suggested for larval frog retina as well (Reh and Tully, 1986). According to this view, maximum proliferation would be associated with maximum cell loss (at 5–6 days postbulbectomy), and the elevated level of proliferation that is maintained in the chron-

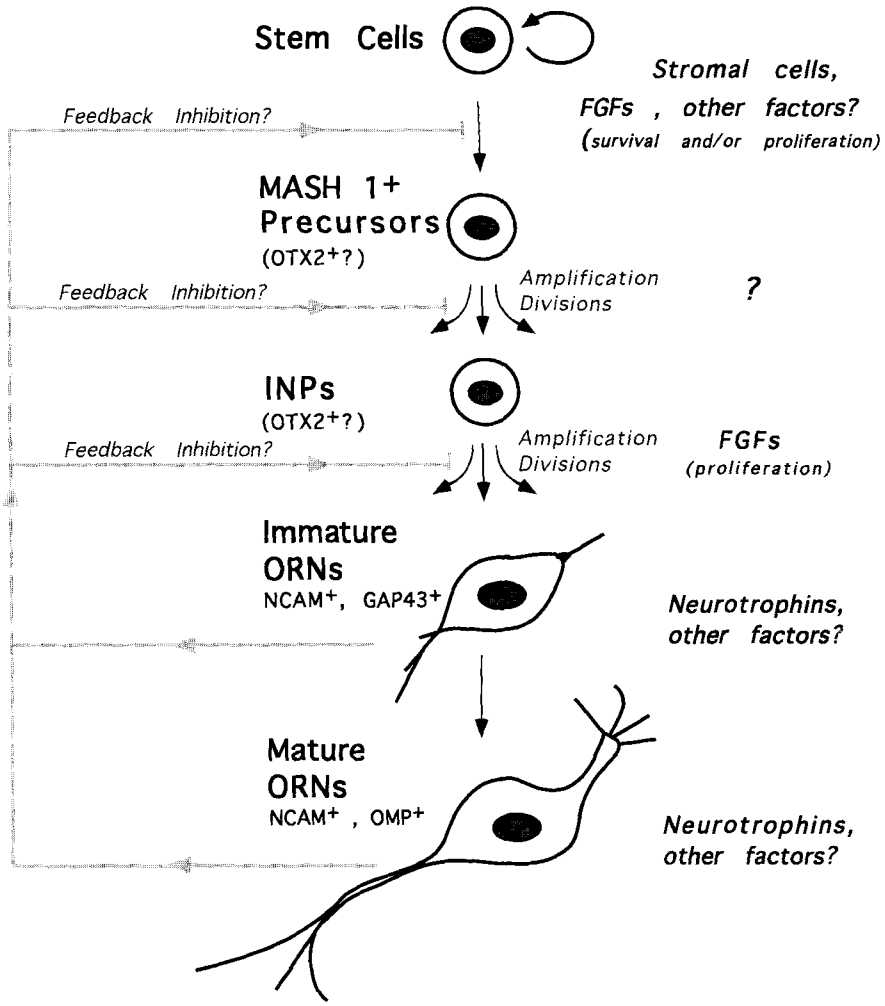


Figure 9 Cell interactions regulating neurogenesis and cell death in the olfactory epithelium. Evidence in support of this model is discussed in detail in the text.

ically bulbectomized OE would be associated with the reduced number of ORNs that are maintained in the OE in this condition.

The results of our studies on apoptosis in the OE suggest the possibility of an additional relationship between neuronal death and precursor proliferation in the OE, however. The peak of apoptosis of OE neuronal cells occurs 2 days postbulbectomy (Fig. 6), preceding both maximum cell loss and the peak of induced ^{3}H-TdR incorporation by 3–4 days. We know that the genesis of ORNs proceeds through at least three precursor cell stages (the stem cell, the MASH1$^+$ cell, and the INP; DeHamer et al., 1994; Gordon et al., 1995). If the signal for induced neurogenesis following bulbectomy acts on an early progenitor cell, perhaps the stem cell, then the peak in proliferation at 5–6 days postbulbectomy that is observed could simply reflect expansion of transit amplifying cells (MASH1$^+$ cells and/or INPs) in response to an early mitogenic stimulus.

This in turn suggests the interesting possibility that actively dying ORNs may provide a positive signal regulating bulbectomy-induced neurogenesis in the OE. In some cells, including neurons, an early step in the apoptotic pathway is the expression of cysteine proteases similar to interleukin-1β-converting enzyme (ICE), an enzyme which cleaves the inactive IL-1β precursor protein to generate the active cytokine (Yuan et al., 1993; Miura et al., 1993; Kumar et al., 1994; Gagliardini et al., 1994). If ORNs express ICE-like enzymes while undergoing apoptosis, such enzymes could potentially activate factors that might

then act as positive regulators of proliferation by early progenitors in the ORN lineage.

CONCLUSIONS

A summary of some of our current ideas concerning the regulation of neurogenesis and cell death in the OE is provided in Figure 9. This model proposes three stages of proliferating neuronal precursors in the ORN lineage: a self-renewing stem cell (for which no molecular marker yet exists); and two stages of neuronal transit amplifying cells, MASH1-expressing cells, and their progeny, the INPs (Gordon et al., 1995). Both types of amplifying precursors may express *Otx2* INPs giving rise to immature ORNs, which are postmitotic cells that express NCAM and GAP43 (Calof and Chikaraishi, 1989; Verhaagen et al., 1990). Immature ORNs undergo further differentiation to become mature ORNs, which express both NCAM and olfactory marker protein (OMP; Margolis, 1980) but not GAP43 (Verhaagen et al., 1990); both immature and mature ORNs appear to be at least partially dependent on neurotrophins for their survival (Holcomb et al., 1995). Our initial culture studies on isolated OE progenitor cells suggest that the neuronal stem cell of the OE may be dependent upon stroma-derived factors, which may include FGFs (DeHamer et al., 1994; J. S. Mumm, J. Shou, and A. L. Calof, submitted). At a later stage in the lineage, FGFs appear to regulate the amplification divisions of INPs (DeHamer et al., 1994). Additional factors acting on the different stages of this lineage have yet to be identified, although feedback inhibition by ORNs is one of the proposed mechanisms for regulating the division of precursors.

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