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The Neuronal Stem Cell of the Olfactory Epithelium

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ABSTRACT: The vertebrate olfactory epithelium (OE) is a system in which behavior of neuronal progenitor cells can be observed and manipulated easily. It is morphologically and functionally similar to embryonic germinal neuroepithelia, but is simpler in that it produces large numbers of a single type of neuron, the olfactory receptor neuron (ORN). The OE is amenable to tissue culture, gene transfer, and *in vivo* surgical approaches, and these have been exploited in experiments aimed at understanding the characteristics of OE neuronal progenitor cells. This has led to the realization that the ORN lineage contains at least three distinct stages of proliferating neuronal progenitor cells (including a stem cell), each of which represents a point at which growth control can be exerted. Neurogenesis proceeds continually in the OE, and studies *in vivo* have shown that this is a regulated process that serves to maintain the number of ORNs

at a particular level. These studies suggest that OE neuronal progenitors—which are in close physical proximity to ORNs—can “read” the number of differentiated neurons in their environment and regulate production of new neurons accordingly. Putative neuronal stem cells of the OE have been identified *in vitro*, and studies of these cells indicate that ORNs produce a signal that feeds back to inhibit neurogenesis. This inhibitory signal may be exerted at the level of the stem cell itself. Recent studies to identify this signal, as well as endogenous stimulatory signals that may be important in regulating OE neurogenesis, are also discussed. © 1998 John Wiley & Sons, Inc. *J Neurobiol* 36: 190–205, 1998

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In the mammalian nervous system, proliferation of neuronal progenitor cells and differentiation of their progeny into neurons—the process known as neurogenesis—appears to be under tight control: Progenitor cell proliferation occurs rapidly at first and then more slowly, and in most regions, permanently ceases toward the end of development (Kauffman, 1968; Caviness et al., 1995). To build a properly functioning nervous system, it is important not only

that neurons be produced, but that they be produced in precise numbers in specific locations. How, and at what cellular stage(s), is this growth control exerted in the developing nervous system? Recent evidence from studies of neurogenesis in the olfactory epithelium (OE) indicates that such regulation may be exerted at the very earliest stages in the neuronal lineage, perhaps at the level of the neuronal stem cell itself.

The OE of the nasal cavity can be divided into three major compartments, illustrated in Figure 1: the apical compartment, which contains the somata of the supporting or sustentacular cells (Hempstead and Morgan, 1983); the basal compartment, which lies adjacent to the basal lamina and contains both “horizontal” (flat, cytokeratin-expressing) basal cells and “globose” (round, cytokeratin-negative) basal cells (Graziadei and Monti Graziadei, 1979; Calof and Chikaraishi, 1989); and the middle com-

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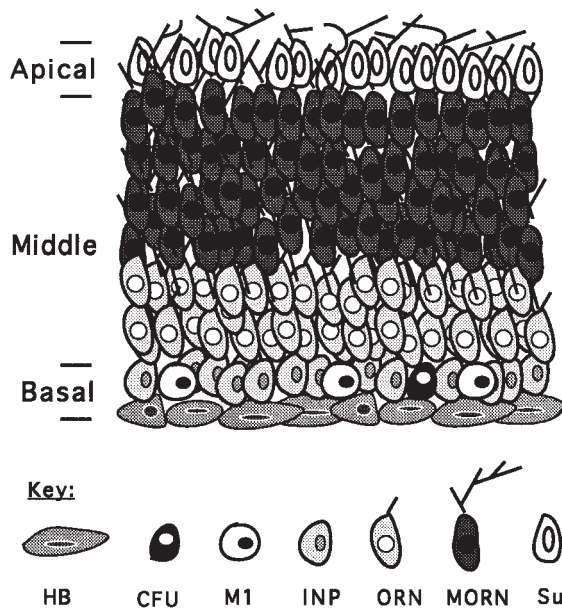


Figure 1 The three major cell compartments in the olfactory epithelium and the relative positions of different cell types within them. The olfactory epithelium consists of three major cell compartments: The apical compartment, which contains the sustentacular cells (Su); the middle compartment, which contains both immature (ORN: NCAM⁺, OMP⁻) and mature (MORN: NCAM⁺, OMP⁺) olfactory receptor neurons; and the basal compartment, which contains both “horizontal” basal cells (HB: keratin⁺), lying adjacent to the basal lamina, and the “globose” basal cells, which lie above them. The globose basal cells are a heterogeneous population, consisting of neuronal colony-forming cells (CFU), MASH1-expressing neuronal progenitors (M1), and immediate neuronal precursors (INP). These cell types and their roles in olfactory neurogenesis are discussed in the text.

partment, which contains the primary sensory neurons [olfactory receptor neurons (ORNs)] that transmit the sensation of smell from the nose to the olfactory bulb of the brain. ORNs can be distinguished from other cell types in the epithelium by their expression of the neural cell adhesion molecule (NCAM), antibodies to which recognize all postmitotic neurons in the epithelium (Calof and Chikaraishi, 1989; DeHamer et al., 1994); ORNs that have fully matured express the cytoplasmic protein olfactory marker protein (OMP), a molecule that is unique to these cells (Margolis, 1980).

Like the keratinizing epithelium of the skin, the OE is in direct contact with the environment, and ORNs are continually lost owing to disease or injury. This is likely to be the cause of the ongoing turnover of ORNs accompanied by compensatory neurogenesis observed in adult animals (e.g., Grazi-

adei, 1973; Moulton, 1974; Graziadei and Monti Graziadei, 1978), as the lifespan of ORNs in animals protected from environment (respiratory) insult is dramatically lengthened (Hinds et al., 1984). It is in its capacity to replace lost neurons in the adult that the OE differs from most other regions of the nervous system, but this ability to regenerate its characteristic differentiated cell type is a functional similarity between the OE and regenerating tissues such as skin, liver, muscle, or blood. As with those other tissues, the presence of stem cells in the OE has long been hypothesized to be the basis of its regenerative capacity.

In vivo studies performed by a number of laboratories have contributed to the realization that neurogenesis in the OE is a highly regulated process, serving to maintain the number of ORNs at a particular level, and such studies have provided support for the existence of a neuronal stem cell in the epithelium. As already discussed, in normal animals, in which ORNs are constantly dying in low numbers (owing to disease or environmental insult), a low level of production of new ORNs is constantly replacing them. If surgical or chemical manipulations are used to eliminate large numbers of ORNs abruptly, the production of new neurons is markedly up-regulated and the epithelium, depending on the extent of insult, can be nearly completely restored. In one such manipulation, one olfactory bulb (the synaptic target of ORNs) is removed from the brain of an adult rodent. When this is done, nearly all ORNs in the OE on the side that innervated that bulb die (Costanzo and Graziadei, 1983; Holcomb et al., 1995). As ORNs die and the OE degenerates (decreases in thickness), neuronal progenitor cells in the epithelium proliferate and replace the lost ORNs (Schwartz-Levey et al., 1991; Gordon et al., 1995).

Such *in vivo* anatomical studies have made it clear that the hypothesized stem cell is likely to reside in the basal compartment of the OE in adult vertebrates (Moulton and Fink, 1972; Graziadei, 1973; Graziadei and Monti Graziadei, 1979; Camara and Harding, 1984; Hinds et al., 1984; Mackay-Sim and Kittel, 1991; Schwartz-Levey et al., 1991; Gordon et al., 1995). Retroviral lineage analyses have confirmed the existence of neuronal progenitors in basal OE and have also contributed to the idea, derived from developmental and immunohistochemical studies of OE, that both horizontal basal cells and sustentacular cells are not part of the neuronal lineage (Klein and Graziadei, 1983; Mulvaney and Heist, 1971; Matulionis, 1976; Caggiano et al., 1994). However, these types of studies alone have not been adequate to define precursor-

progeny relationships in the ORN lineage, nor for identifying and characterizing the stem cell of the OE. The most significant progress in these areas has come from a combinatorial approach in which molecular markers for particular cell types are used in conjunction with tissue culture studies, *in vivo* surgical paradigms, and the analysis of mutant and transgenic mice.

MULTIPLE CELL STAGES IN THE NEURONAL LINEAGE OF THE OLFACTORY EPITHELIUM

Although the OE was perceived initially as a relatively simple neuroepithelium, ongoing studies of neurogenesis have revealed that the ORN lineage is much more complex than was previously appreciated. In our laboratory, this understanding has evolved during the course of ongoing *in vitro* studies of molecular regulation of neurogenesis in mouse OE (Calof et al., 1995; Mumm et al., 1996). Initially, explant cultures of OE purified from embryonic day 14–15 (E14–15) mouse embryos were developed (Calof and Chikaraishi, 1989). To facilitate these studies, we identified antibody markers that recognize two major cell types in the OE, *in vitro* and *in vivo*: antibodies to NCAM, which mark postmitotic, differentiated ORNs; and a commercial antiserum to keratins, which marks the horizontal basal cells (Calof and Chikaraishi, 1989). In OE explant cultures, the pieces of explanted epithelium adhere to appropriate culture substrata (Calof and Lander, 1991), and within hours a population of cells sorts out from the main body of the explant (in which keratin-expressing horizontal basal cells remain) and begins to migrate out onto the substratum (Calof and Chikaraishi, 1989; Calof and Lander, 1991). Virtually all of these migratory cells are either NCAM⁺ ORNs or proliferating cells that incorporate [³H]thymidine (³H-TdR). Over the course of 2 days in culture, the ORNs and proliferating cells continue to migrate, during which time nearly every proliferating cell divides once to give rise to two daughter cells that both differentiate into NCAM-expressing ORNs. This process was followed by pulse-labeling cells with ³H-TdR and following their acquisition of the ORN-specific NCAM marker (Calof and Chikaraishi, 1989; DeHamer et al., 1994). These and other experiments have demonstrated that neurogenesis occurs efficiently in OE cultures, and served to identify the direct progenitor of ORNs, which we have called the immediate neuronal precursor (INP) (Fig. 1). *In vivo* anatomical studies subsequently confirmed our hypothesis that

the INPs are the immediate progenitors of ORNs, and placed INPs among the cells referred to as “globose” basal cells of the OE (Mackay-Sim and Kittell, 1991).

Studies by others suggested that INPs *in vivo* could undergo two to three rounds of division before their progeny differentiated into ORNs (Mackay-Sim and Kittell, 1991); however, we initially observed only one round of INP division in our cultures. By screening for molecules that prolong INP proliferation in OE cultures, we were able to identify members of the fibroblast growth factor family (FGFs), particularly FGF-2, as stimulators of INP divisions (DeHamer et al., 1994). That multiple divisions of INPs occur in FGF-2 was demonstrated conclusively using sequential labeling with bromodeoxyuridine (BrDU) and ³H-TdR to specifically mark ORNs that had been generated as a result of two successive rounds of cell division in culture. We found that the incidence of such double-labeled ORNs is four to five times greater in FGF-2-treated cultures than in controls (DeHamer et al., 1994). Importantly, FGFs do not alter the fate of INPs; virtually all INPs still give rise to ORNs, even with FGFs present. Thus, as committed neuronal progenitor cells capable of undergoing a limited number of divisions in response to exogenous factors, INPs fit the description of “transit amplifying cells,” a term used to describe intermediate-stage progenitors in other lineages (e.g., the hematopoietic lineage) (Hall and Watt, 1989; Potten and Loeffler, 1990; DeHamer et al., 1994).

Further studies revealed additional complexity in the ORN lineage: In this work, we were interested in understanding why mice with disruption of both alleles of the gene encoding *Mammalian Achaete Scute Homolog 1* (*Mash1*) show a near total absence of ORNs, as well as certain autonomic and enteric neurons (Guillemot et al., 1993). Using explant cultures of OE obtained from wild-type animals, we found that MASH1 is expressed by neuronal progenitor cells at a distinct stage in the ORN lineage, a stage upstream of INPs (Gordon et al., 1995). Evidence supporting this conclusion came partly from observing the rapid disappearance of MASH1⁺ cells from OE explant cultures; this disappearance appears to reflect the division of MASH1⁺ cells to generate MASH1-negative INPs (Gordon et al., 1995). Additional studies in which olfactory bulbectomy was used to induce neurogenesis in adult OE *in vivo* provided data on how changes in the numbers and proliferative states of MASH1⁺ cells correlate with changes in overall neurogenesis; the results of these experiments strongly suggest that MASH1-expressing cells give

rise to INPs, but are not the self-renewing stem cells of the OE (Gordon et al., 1995). Instead, the kinetics of proliferation of MASH1⁺ cells indicate that they, like INPs, act as neuronal transit amplifying cells (Gordon et al., 1995). In addition, MASH1⁺ cells, like INPs, are found in the basal compartment of adult OE (Fig. 1).

To see if we could gain further insights into the characteristics of neuronal stem cells in the OE, we analyzed OE in E14–15 *Mash1*^{-/-} embryos to determine why absence of *Mash1* gene function might result in cessation of ORN development. We found that few NCAM⁺ ORNs ever appear in *Mash1*^{-/-} embryos, suggesting that the lack of ORNs at birth apparently reflects a defect in ORN production, rather than ORN survival. Although the OE of *Mash1*^{-/-} embryos is abnormally thin, it still contains a substantial number of NCAM⁺ cells (presumptive neuronal progenitors). Using TUNEL staining for DNA fragmentation as a criterion for apoptotic cell death (Gavrieli et al., 1992), we found that apoptosis is strikingly elevated in the OE of E14 *Mash1* null embryos (Fig. 2): There are ~55 [55.87 ± 13.71, mean ± standard deviation (S.D.)] apoptotic cells per mm OE in *Mash1*^{-/-} animals, compared to ~7 (7.72 ± 2.87 for *Mash1*^{+/+}; 7.01 ± 0.54 for *Mash1*^{+/-}) in normal animals. Furthermore, by performing double-label studies in which sections of *Mash1*^{-/-} OE were stained for both NCAM (an ORN-specific marker) and TUNEL (to detect apoptotic cells), we found that the cell death taking place in *Mash1*^{-/-} OE is not in the few NCAM⁺ cells that escape the genetic lesion. Rather, virtually all (98.7%) apoptotic cells in *Mash1*^{-/-} OE are NCAM-negative cells (Calof et al., 1996). The data suggest that in the absence of *Mash1* function, neuronal stem cells produce neuronal progenitor cells (which normally would be MASH1⁺), but most of these progenitor cells die without generating ORNs.

These findings predict that at least some progenitors of ORNs (presumably neuronal stem cells, and possibly their immediate progeny) are present and mitotically active in *Mash1*^{-/-} OE. If so, it should be possible to rescue neurogenesis by expressing a *Mash1* transgene in progenitor cells of *Mash1*^{-/-} OE. To accomplish this, we made explant cultures of OE from embryos derived from mating *Mash1* heterozygotes (homozygous null animals die around the time of birth), and used NCAM expression by cells in the explants as a criterion for neurogenesis. OE explants from *Mash1*^{-/-} embryos grow and are viable, but after a day in culture, only ~4% (3.6 ± 3.5%) have generated NCAM⁺ ORNs, whereas at the same time, ~95% (94.8 ± 1.1%) of explants

from wild-type and heterozygote littermates have done so.

To test whether neurogenesis could be restored in *Mash1*^{-/-} OE, a *Mash1* retroviral expression vector was constructed by inserting the cDNA for mouse *Mash1* (Guillemot and Joyner, 1993) into LIGNS, a retroviral vector giving high titers and levels of expression due to retention of endogenous retroviral *gag* sequences (Lillien, 1995). In preliminary experiments, cultures of OE explants from *Mash1*^{+/+}, *+/+*, and *-/-* embryos were plated onto feeder layers of growth-arrested *Mash1*-LIGNS Ψ2 (virus-packaging) cells; as a control, half of the tissue from each embryo was plated onto Ψ2 cells packaging the LIGNS vector minus the *Mash1* insert. Explants were grown for 28 h and then scored for NCAM immunoreactivity to determine the level of neurogenesis. Exposure of *Mash1*^{-/-} OE to the *Mash1*-LIGNS virus resulted in a twofold increase in the percentage of explants showing a high level of neurogenesis (“high NCAM score”), while exposure to the *Mash1*-LIGNS virus had no apparent effect on neurogenesis in wild-type or heterozygote explants [Fig. 3(A)]. These results indicate that the defect in olfactory neurogenesis resulting from absence of the *Mash1* gene can be at least partially rescued by forcing expression of *Mash1* in proliferating cells in *Mash1*^{-/-} OE, lending further support to the idea that neuronal stem cells are still present.

In the hematopoietic system, if multipotent progenitors are rescued from cell death by overexpression of the *bcl-2* gene, they go on to differentiate in the absence of added growth factors (Fairbairn et al., 1993). If the ultimate function of MASH1 is to regulate expression of genes required for neuronal progenitor survival, then by analogy with the hematopoietic system, it may be possible to bypass the *Mash1* null phenotype by “artificially” supporting the survival of *Mash1*^{-/-} progenitors. To test this hypothesis, we generated an LIGNS-based retroviral vector to express *bcl-2*, a proto-oncogene that blocks apoptosis in a variety of cells, including neurons (Hockenbery et al., 1990; Davies, 1995). Ψ2 cells that package the *bcl2*-LIGNS vector were generated; growth-arrested feeder layers made from them; and OE explants from *Mash1*^{+/+}, *+/+*, and *-/-* embryos were plated onto these feeders and grown and analyzed as described above. Expressing Bcl2 in *Mash1*^{-/-} OE had an even greater positive effect on neurogenesis than did expressing MASH1 [Fig. 3(A,D)]: three times as many *Mash1*^{-/-} explants had a high NCAM score when exposed to the *bcl2*-LIGNS virus as did when exposed to control virus. These preliminary findings suggest that preventing progenitor cell death in *Mash1*^{-/-} OE

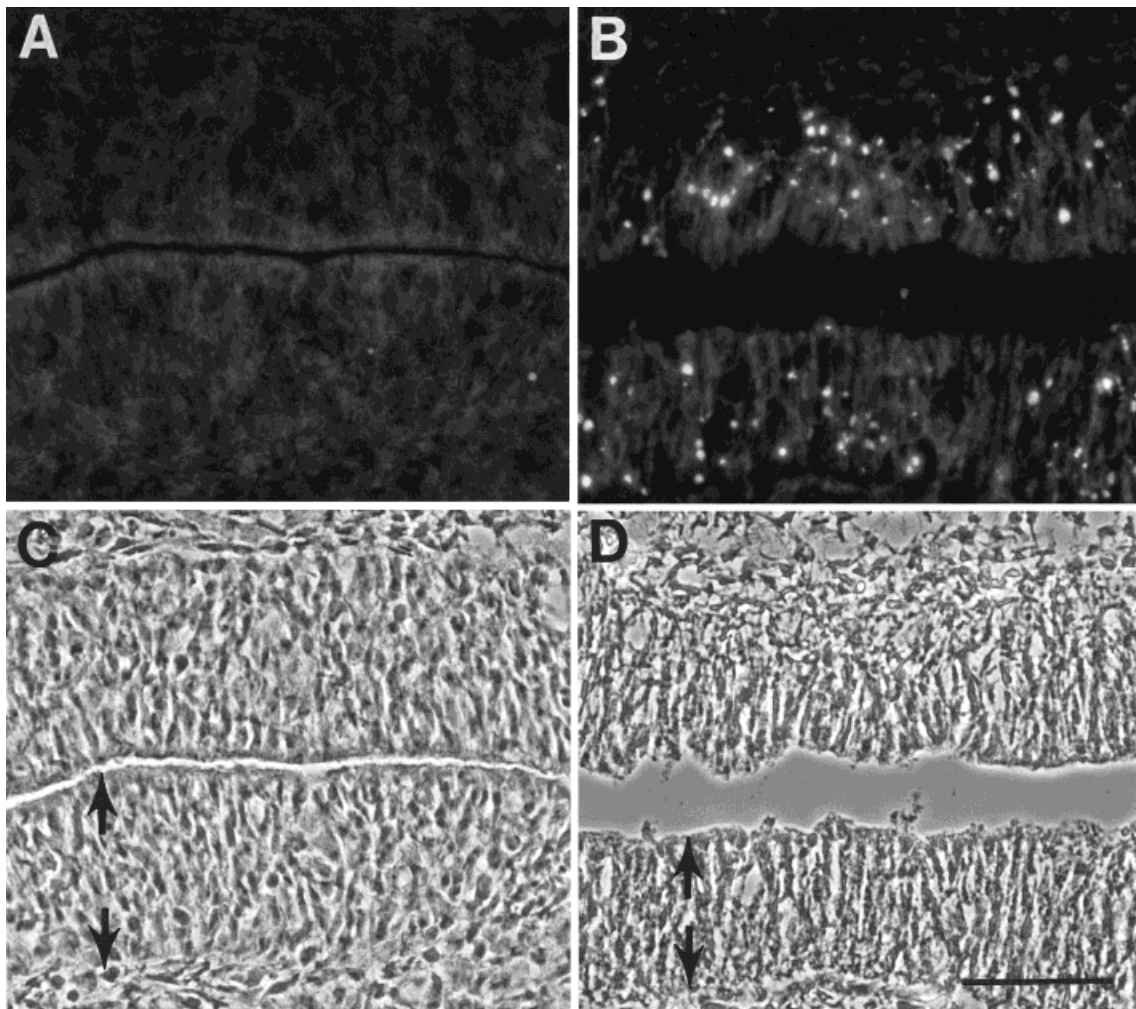


Figure 2 Increased cell death in the olfactory epithelium of *Mash1*^{-/-} mice. E14 embryos from *Mash1* heterozygous crosses were fixed by freeze substitution and sectioned at 14 μm on a cryostat. TUNEL reactions to detect apoptotic cells were performed as described in Holcomb et al. (1995), with Texas red-conjugated Neutralite avidin (Molecular Probes; 1:200 dilution) used to detect incorporation of biotin-16-dUTP. (A,C) In the wild-type embryo, there is virtually no cell death in the epithelium at this age. (B,D) There are many apoptotic cells in the OE of *Mash1*^{-/-} mice (B), and the epithelium itself is much thinner owing to the absence of ORNs (D, arrows). Bar = 100 μm .

is sufficient to restore neurogenesis, although the possibility that this effect might result from enhanced proliferation by Bcl2-overexpressing progenitors has yet to be ruled out (A. L. Calof, P. C. Rim, and J. Shou, unpublished results). Despite this caveat, however, these studies reinforce the hypothesis that neuronal stem cells are still present in *Mash1*^{-/-} animals. In addition, they suggest that a major function of MASH1 in the ORN lineage may be to control (directly or indirectly) the expression of genes that play roles in regulating the survival of neuronal progenitor cells.

Among the downstream targets of MASH1 may be other transcription factors. For example, Cau and

colleagues recently showed that *Math4C/neurogenin1*, which like *Mash1* encodes a basic helix-loop-helix transcription factor, is expressed by a population of progenitor cells in the OE *in vivo*. This progenitor cell population is separate from *Mash1*-expressing progenitors, but the data indicate that *neurogenin1* expression is genetically downstream of MASH1, since *neurogenin1* is not expressed in the OE of *Mash1*^{-/-} embryos (with the exception of a small ventrocaudal domain of OE that appears to be *Mash1* independent). Thus, these authors have postulated that *neurogenin1* is expressed by the INP, an interesting hypothesis that remains to be tested *in vitro* (Cau et al., 1997).

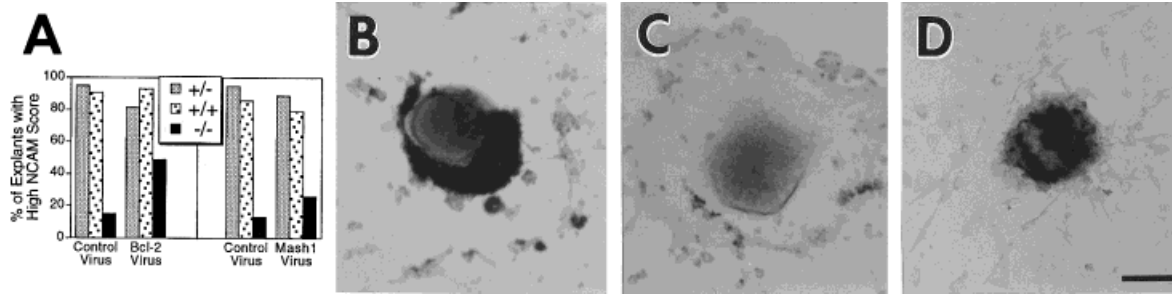


Figure 3 Restoration of neurogenesis in *Mash1*^{-/-} olfactory epithelium. (A) OE explants from *Mash1*^{+/+}, *+/+*, or *-/-* embryos were plated separately in serum-free medium (with 5 mg/mL BSA) (Calof et al., 1995) onto growth arrested (3300 rad), confluent Ψ2 feeder cell layers producing either control, Bcl2, or Mash1 retroviruses. To distinguish the genotype of the OE tissue used in each culture, DNA was prepared from unused tissue from each embryo and primers to the *neo* coding region in the *Mash1* targeting vector (present only in *Mash1* nulls and heterozygotes) and to endogenous *Mash1* (present only in wild types and heterozygotes) were used to amplify DNA in PCR reactions. After 28 h *in vitro*, cultures were fixed in 10% formalin in phosphate-buffered saline for 10 min, then reacted with H28 monoclonal anti-NCAM (DeHamer et al., 1994) and visualized with horseradish peroxidase-conjugated goat anti-rat immunoglobulin G (Chemicon; 1:300 dilution). Explants that possessed >10 NCAM-expressing neurons were given a “high NCAM” score, and the percentage of total explants receiving a high NCAM score was calculated for all cultures from animals of a given genotype. (A) Exposing *Mash1* null OE to either Bcl2 or Mash1 retroviruses resulted in a partial restoration of neurogenesis (“High NCAM Score”; *-/-*, black bars) compared to heterozygous and wildtype OE (*+/+*, *+/+*), and increased neurogenesis two- to three-fold over that seen with control virus. (B–D) Photomicrographs of explants grown for 28 h on Ψ2 feeder layers, then stained with anti-NCAM to detect production of ORNs. (B) Wild-type explant, control virus. (C) *Mash1*^{-/-} explant, control virus. (D) *Mash1*^{-/-} explant, Bcl2 virus. Note high level of NCAM staining (dark brown) in (B) and (D), but not (C). Bar = 100 μm.

Another class of molecules likely to be downstream targets of *Mash1* is receptors for neuronal trophic factors. Our previous studies had indicated that neurotrophin-3 (NT-3), the neurotrophin ligand for the tyrosine kinase receptor (TrkC), promotes survival of embryonic OE neuronal cells *in vitro* and that TrkC is expressed in neuronal cells, particularly ORNs, in neonatal OE (Holcomb et al., 1995). Because TrkC expression is detectable in the OE at such an early age, and because NT-3 has been shown to promote survival of neuronal progenitor cells in other tissues (Birken et al., 1993; DiCicco-Bloom et al., 1993), we examined TrkC expression in OE of E14–15 *Mash1*^{-/-} embryos. As shown in Figure 4, there is widespread TrkC immunoreactivity in wild-type OE, but none in OE of *Mash1*^{-/-} littermates, suggesting that *trkC* lies genetically downstream of *Mash1*. Interestingly, the pattern of immunoreactivity, showing TrkC⁺ cells within most layers of E14 OE, suggests that, within OE of normal embryos, both neuronal progenitor cells and ORNs express TrkC. Expression of this receptor

may be an important developmental event regulating progenitor cell survival in the OE.

Thus, *in vivo* and *in vitro* analyses of wild-type and mutant mice have led to the realization that the ORN lineage is much more complex than had previously been appreciated, with at least two distinct types of proliferating transit amplifying progenitors—the MASH1⁺ progenitor and the INP—interposed between the ORN and the stem cell (Gordon et al., 1995). In addition, these studies suggest that an important consequence of lineage complexity may be that it provides opportunities for cell number to be regulated at each cell stage in the lineage (Calof, 1995).

WHAT IS THE IDENTITY OF THE OE NEURONAL STEM CELL?

Our initial clue that neuronal stem cells might be present and functioning in OE cultures was derived from our studies of FGF actions on olfactory neurogenesis. This work showed that FGFs can act in

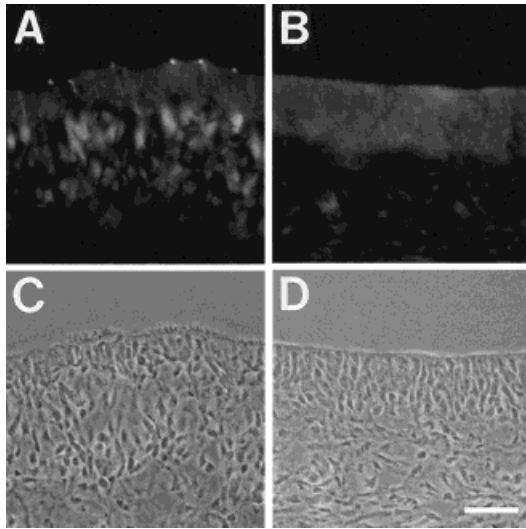


Figure 4 Absence of TrkC immunoreactivity in olfactory epithelium of *Mash1*^{-/-} embryos. Heads of E15 *Mash1*^{+/+}, ^{+/-}, or ^{-/-} embryos were fixed by immersion for 3 h in 4% paraformaldehyde in phosphate buffer, equilibrated in 15% sucrose in phosphate-buffered saline (PBS), embedded in 7.5% gelatin in 15% sucrose/PBS, and sectioned at 14 μ m on a cryostat. Sections were rinsed in PBS/0.1% Triton X-100, incubated in rabbit antiserum to TrkC (anti-TrkCin2, 1:500 dilution in 10% calf serum/PBS) (Holcomb et al., 1995) overnight in the cold, and anti-TrkC binding was visualized with Cy3-conjugated donkey anti-rabbit immunoglobulin G (1:200 dilution; Jackson Immunoresearch). (A,C) TrkC immunoreactivity is found in scattered cells throughout the epithelium of wild-type embryos. Some ORNs, with apical dendrites extending to the luminal surface of the OE, are also stained. (B,D) TrkC immunoreactivity is completely absent in the OE of *Mash1*^{-/-} embryos. Bar = 25 μ m.

two ways to promote neurogenesis in the OE: by promoting proliferation of INPs (discussed above), and, at longer periods in culture, by increasing proliferation and/or survival of rare early ORN progenitors, putative neuronal stem cells (DeHamer et al., 1994). To demonstrate these longer-term effects of FGFs on OE neurogenesis, OE explants were grown for 48–96 h in the presence of FGF-2 or no growth factor, and exposed to ³H-TdR for the last 24 h of culture. Labeling indices were determined for each explant by counting the total number of ³H-TdR-labeled cells (assessed by emulsion autoradiography) surrounding each explant; this number was normalized to an average explant size (Fig. 5). These data were plotted as frequency histograms to reveal the percentages of explants with labeling indices falling within different ranges. As Figure 5 shows, at 72 and 96 h in culture, FGF-2 allowed a reproducible, small subset of OE explants (5–8%

of total explants) to continue to generate large numbers of proliferating neuronal progenitor cells (“>35” bin); whereas in the absence of FGF2, no explants had high labeling indices. Confirmation that these ³H-TdR-labeled cells were neuronal progenitors was obtained by performing pulse-chase ³H-TdR incorporation experiments and demonstrating the presence of newly generated, NCAM-expressing ORNs within them (DeHamer et al., 1994).

These results indicate that a small fraction of explants (5–8%) undergoes continuous ORN production for up to 4 days *in vitro* in the presence—but not in the absence—of FGF-2. The data are suggestive that explants in this “outlier” fraction contain within them rare cells of high proliferative potential, cells that are absent in the majority of explants, but which are revealed when explants are cultured in the presence of FGF-2. The apparent rarity of such cells (estimated to be about 1 in 2500 of migratory cells in explant cultures), coupled with the observation that they are capable of extended neurogenesis in culture, suggested to us that they are progenitors that lie significantly upstream of INPs in the ORN lineage—perhaps the neuronal stem cells of the OE (DeHamer et al., 1994).

Because such studies on OE explants gave only indirect evidence for the presence of a neuronal stem cell in OE cultures, we decided to take a more direct approach to attempt to isolate and characterize these cells. For these experiments, we developed methods to purify neuronal progenitor cells from the OE and characterize their development *in vitro*. This was accomplished by immunological panning, in which anti-NCAM-treated petri dishes were used to selectively remove postmitotic ORNs from a dissociated neuronal cell fraction containing only NCAM⁺ ORNs and NCAM⁻ progenitors (Calof and Lander, 1991; Holcomb et al., 1995). This procedure typically yields a preparation that consists of >96% NCAM-negative, presumptive ORN progenitors (Mumm et al., 1996).

Quantitatively, this purified progenitor cell fraction turns out to contain mostly INPs, as was expected, since INPs constitute the vast majority of neuronal progenitor cells in the OE (Calof and Chikaraishi, 1989; DeHamer et al., 1994; Gordon et al., 1995). Indeed, most of these purified progenitors behave as neuronal transit-amplifying cells when they are plated in isolated culture on defined substrata in serum-free medium: Under these conditions, >95% of purified progenitors rapidly give rise to differentiated, NCAM-expressing ORNs, all of which then die within a few days, presumably owing to the absence of target tissue or appropriate

trophic support (Calof and Chikaraishi, 1989; DeHamer et al., 1994; Gordon et al., 1995; Holcomb et al., 1995; Mumm et al., 1996). In an effort to enhance survival and/or proliferation of neuronal progenitors, we turned to culturing them over monolayers of feeder cells isolated from the stroma that lies subjacent to the OE, and with which OE progenitors are normally associated *in vivo*. For these experiments, OE progenitors were purified from Rosa26 mice, which express a *lacZ* transgene in every cell (Friedrich and Soriano, 1991); this enabled us to use X-gal histochemistry or immunocytochemistry using antibodies to bacterial β -galactosidase, to detect progenitors and their progeny when cultured on stroma derived from outbred CD-1 mice (Mumm et al., 1996).

When this is done, the majority of purified

Rosa26-derived progenitors still behave like INPs: They rapidly differentiate into NCAM-expressing neurons and then die. However, after 6–7 days *in vitro* in the coculture condition, about 1 in 1000 progenitors goes on to develop into morphologically homogeneous, proliferative colonies, as shown in Figure 6. Despite the existence of variation in cell morphology among colonies [Fig. 6(A–D)], cells within a colony always have a similar morphology, which suggests that colonies are derived from clonal expansion; this was supported by experiments in which a number of colonies were inspected periodically for several days, during which time progressive increases in colony size were seen (Mumm et al., 1996). One of these colony types, accounting for about one fourth of the colonies that appear in these cultures, contained tightly clustered cells which possess obvious neurites [Fig. 6(D)]. Indeed, when cultures were stained with antibodies to NCAM, only this colony type was found to contain NCAM-expressing ORNs among its cells [Fig. 6(E)]. To prove that these neuronal colonies actively produce neurons, pulse-chase ^3H -TdR incorporation experiments were performed in progenitor/stroma cocultures to label neurons being generated from 6 to 7.5 days *in vitro*. As shown in Figure 7, many NCAM-expressing neurons (red) in neuronal colonies were also ^3H -TdR labeled (yellow) in such experiments, indicating that ORN production is on-

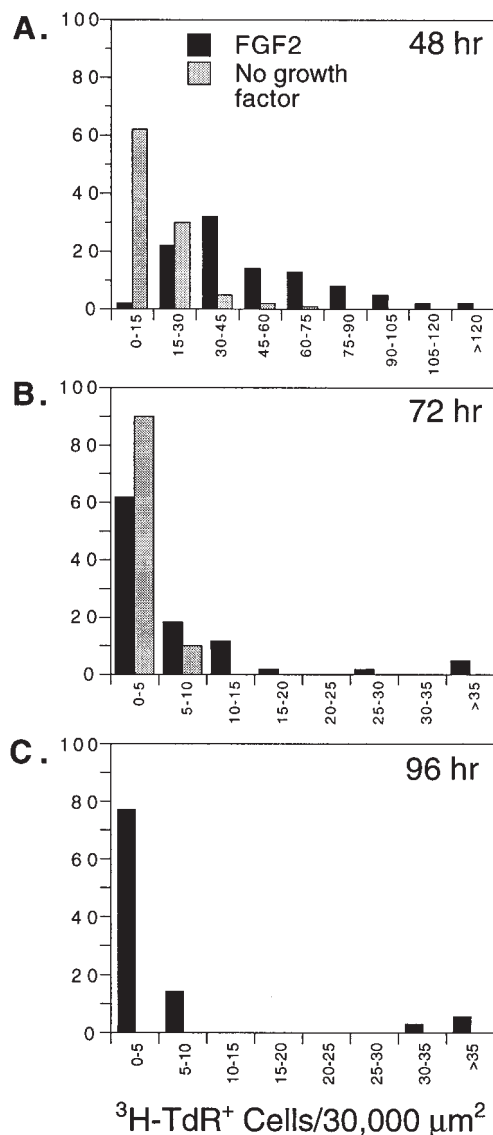


Figure 5 Effects of FGF-2 on distribution of explant labeling indices at late times in culture. OE explants were cultured in the presence or absence of FGF-2 (10 ng/mL) for either 48, 72, or 96 h and were exposed to ^3H -TdR (0.1 $\mu\text{Ci}/\text{mL}$) during the last 24 h of culture. Following autoradiography, the numbers of labeled migratory cells surrounding a large number of explants (see below) were calculated and normalized for each explant to an average explant area of 30,000 μm^2 . Ordinate scale is percentages of explants exhibiting particular ranges of explant labeling indices. (A) Forty-eight-hour cultures in the presence versus absence of FGF-2. One hundred explants were analyzed in each condition. (B) Seventy-two-hour cultures in the presence versus absence of FGF-2. Sixty explants were analyzed in each condition. Note that the bin sizes are smaller than in (A). (C) Ninety-six-hour culture grown in the presence of FGF-2. Thirty-five explants were analyzed. The data indicate that FGF-2 allows a reproducible, small subset of OE explants to continue to generate large numbers of proliferating neuronal progenitor cells at 72–96 h in culture, whereas in the absence of FGF-2, no explants have high labeling indices. (Adapted with permission from DeHamer et al., *Neuron* 13:1083–1097, © 1994, Cell Press.)

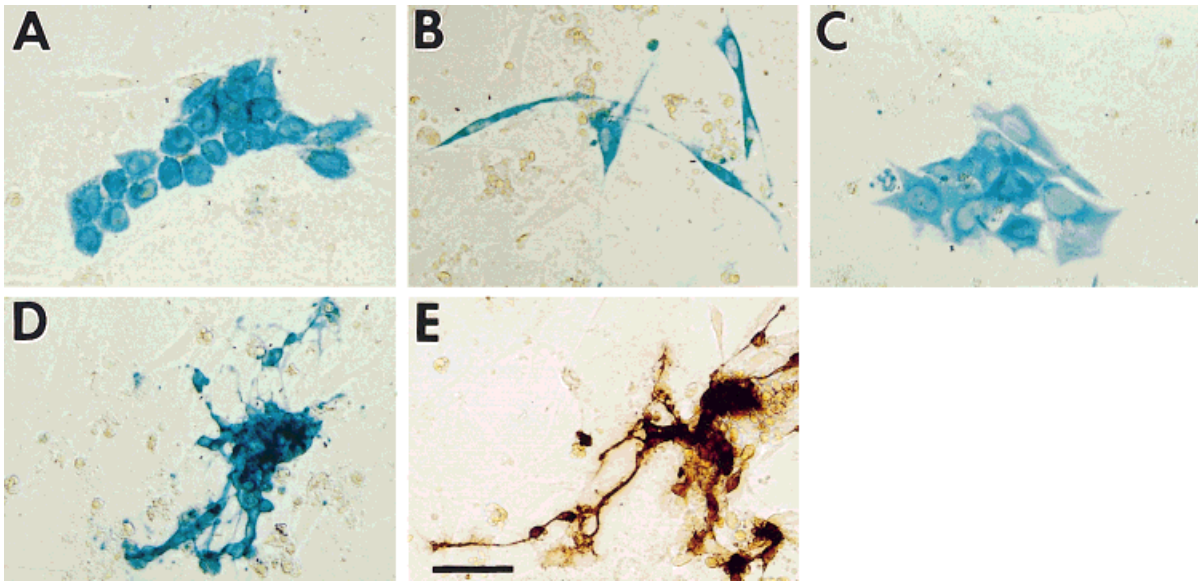


Figure 6 Purified progenitors generate colonies with four distinct cellular morphologies, one of which is neuronal. Rosa26 progenitor cells, purified by immunological panning, were cocultured over monolayers of growth-arrested CD-1 stroma cells in 24-well plates. Cultures were fixed and processed with X-gal [(A–D), blue] or for NCAM immunoreactivity [(E), brown] after 7 days *in vitro*. Four distinct colony morphologies are observed: (A) medium-round cells; (B) spindle-shaped cells; (C) polygonal fibroblastic cells; and (D) small, round cells, many with neuritic processes. A single colony of the small round cell type is shown stained for NCAM in (E). Many NCAM⁺ cells with distinctly neuronal morphology (neurites tipped with growth cones) are observed. This last colony type was termed “neuronal.” Bar = 50 μ m. (Adapted with permission from Mumm et al., *Proc. Natl. Acad. Sci. USA* **93**:11167–11172, © 1996 National Academy of Sciences, U.S.A.)

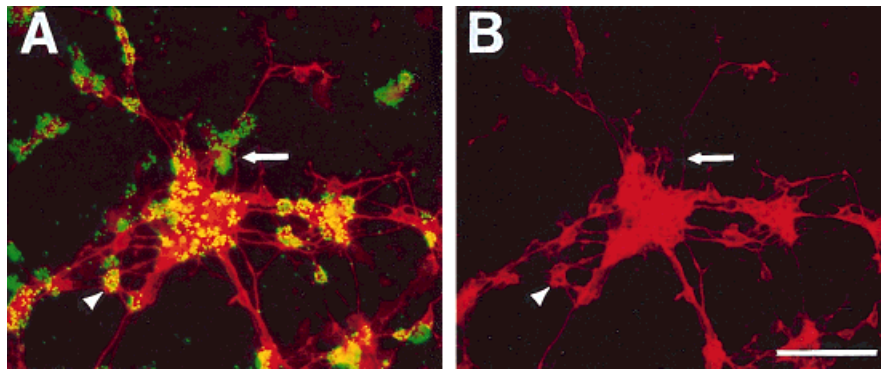


Figure 7 Continual neurogenesis in neuronal colonies at 7 days *in vitro*. Cocultures of purified OE progenitors over growth-arrested stroma were pulsed for 10 h with 1 μ Ci/mL ³H-TdR at 6 days *in vitro*, then chased with cold thymidine (50 μ M) for 24 h prior to fixation and processing for NCAM immunoreactivity and autoradiography. (A) A neuronal colony is shown in a double exposure for NCAM immunoreactivity (red) and silver grains indicating ³H-TdR incorporation. Silver grains appear green if present in an NCAM[−] cell, yellow if present in an NCAM⁺ cell. Some dividing cells have generated postmitotic, NCAM⁺ neurons (arrowhead), but a significant number NCAM[−], ³H-TdR⁺ cells remain (arrow). For clarity, the same colony, exposed for NCAM immunoreactivity alone, is shown in (B). The results indicate that neurons continue to be generated as late as day 7 in culture by proliferating progenitors present in neuronal colonies, and undifferentiated progenitors continue to be generated as well. Bar = 50 μ m (Adapted with permission from Mumm et al., *Proc. Natl. Acad. Sci. USA* **93**:11167–11172, © 1996 National Academy of Sciences, U.S.A.)

going in these colonies for at least a week in culture. Significantly, ^3H -TdR-labeled cells that were NCAM negative were also observed in such neuronal colonies (Fig. 7), indicating that these colonies contain both differentiated ORNs and undifferentiated, proliferating progenitor cells (Mumm et al., 1996).

Our current hypothesis is that neuronal colonies arise from a neuronal colony-forming cell or unit (CFU) similar to CFUs that are thought to be indicative of stem cells in hematopoietic development (Dexter and Spooncer, 1987). We find that neuronal colonies are able to produce differentiated ORNs as late as 13 days *in vitro* (J. Shou, P. C. Rim, and A. L. Calof, unpublished results), and the existence of proliferative, undifferentiated cells within them has been demonstrated as late as 7.5 days *in vitro* (Mumm et al., 1996). These findings are consistent with the presence within neuronal colonies of progenitors with both the capacity for self-renewal and the ability to give rise to a lineage whose end point is a terminally differentiated cell. These are defining characteristics of stem cells (Lajtha, 1979; Hall and Watt, 1989; Potten and Loeffler, 1990).

In some permanently renewing systems, such as the hematopoietic system, stem cells also are characteristically rare (Dexter and Spooncer, 1987). In this light, it is interesting to note that our initial analysis of neuronal stem cell activity in purified progenitor/stroma cell cocultures yielded an estimate of 1 in 3600 progenitors for neuronal CFU frequency (Mumm et al., 1996). Significantly, this is in the range of the estimate for stem cells derived from studies of long-term explant cultures grown in FGF-2 (there the estimate was about 1:2500; see above). It is also on the order of that observed for spleen CFUs isolated from bone marrow (~1 in 3800) (Spooncer et al., 1985), although somewhat lower than estimates of stem cell frequency in epidermis or fetal liver (Jordan et al., 1990; Jones and Watt, 1993). Since the procedures required for purifying progenitors could easily cause damage, and it is virtually certain that we have not yet optimized conditions for culturing neuronal CFUs, we regard this number as a likely underestimate of the true incidence of neuronal stem cells in the OE. The rarity of neuronal colonies does, however, suggest a system dependent upon proliferation of transit amplifying cells for most of its regenerative capacity, and this is in keeping with what is known concerning the multistep nature of neurogenesis in the OE (DeHamer et al., 1994; Gordon et al., 1995). Placing the neuronal colony-forming cell (neuronal CFU) as our current best candidate for the neuronal

stem cell, the neuronal lineage of the OE is diagrammed in Figure 8.

CELL INTERACTIONS REGULATING PROLIFERATION OF PROGENITOR CELLS

Experimental up-regulation of neurogenesis in OE has been a useful paradigm for generating hypotheses concerning cell interactions that regulate this process. As discussed previously, surgical removal of the olfactory bulb on one side of the brain (unilateral olfactory bulbectomy) of an adult rodent causes ORNs in the OE on that side to rapidly undergo cell death; the peak of apoptosis is approximately 2 days following surgery (Holcomb et al., 1995). Neuronal progenitor cells in the ipsilateral OE respond by increasing proliferation, which reaches a peak at about 5–6 days postbulbectomy (Schwarz-Levey et al., 1991; Gordon et al., 1995). There is marked degeneration of the ORN cell layer, which can be quantified by measuring epithelial thickness, and this degeneration follows a time course similar to that of progenitor cell proliferation: Epithelial thickness is at a minimum at about 5 days postbulbectomy (Costanzo and Graziadei, 1983; Schwartz-Levey et al., 1991; Holcomb et al., 1995). As new ORNs are generated in the ipsilateral OE, epithelial thickness increases again to approximately 70% of

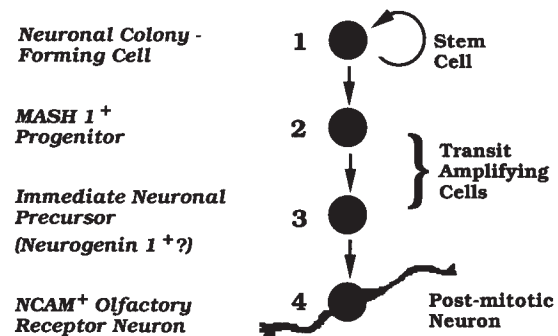


Figure 8 A proposed model of the neuronal lineage in the olfactory epithelium. Our studies have identified three stages of neuronal progenitor cells in the ORN lineage: mammalian achaete-scute homologue-1 (MASH1) expression demarcates cells at the earliest transit-amplifying stage; this stage appears to be interposed between the neuronal stem cell (tentatively identified as the neuronal colony-forming cell or neuronal CFU) and the immediate neuronal precursor (INP). INPs are the last proliferating cells in the ORN lineage: they undergo one or more rounds of division, after which their progeny differentiate into NCAM-expressing ORNs. ORNs are postmitotic cells.

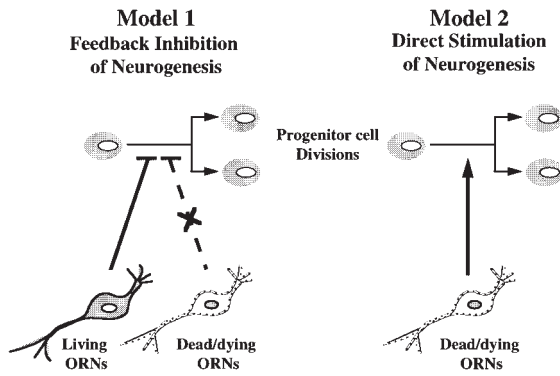


Figure 9 Two models for the regulation of progenitor cell proliferation by olfactory receptor neurons. As proposed in model 1, the feedback inhibition model, living ORNs produce a negative feedback signal that inhibits progenitor cell divisions. When ORNs die, this inhibitory signal is removed, and progenitor cells proliferate and generate new ORNs. In model 2, dying or dead ORNs produce stimulatory factors that promote progenitor cell divisions directly. The two models are discussed in the text.

its original value, and progenitor cell proliferation decreases, but to a level that is elevated over that seen in the contralateral (control) OE (Schwartz-Levey et al., 1991; Gordon et al., 1995; Holcomb et al., 1995).

The temporal relationship of ORN death, cell degeneration, progenitor cell proliferation, and generation of new ORNs suggests two models for how cell interactions in OE could regulate progenitor cell proliferation. These models are diagrammed in Figure 9. In model 1, the feedback inhibition model, proliferation of progenitor cells following death and degeneration of the ORNs overlying them is the result of an interruption of an ORN-derived negative feedback signal that inhibits progenitor cell proliferation. This model is consistent with the observation that maximum proliferation is associated with maximum ORN loss (i.e., maximum epithelial degeneration, at 5–6 days postbulbectomy) and that an elevated level of proliferation is maintained in chronically bulbectomized animal (where a reduced number of ORNs is present in the OE). In model 2, dying or dead ORNs produce a stimulatory signal which directly promotes progenitor cell division. This model is consistent with the results of studies on bulbectomy-induced apoptosis in the OE. Apoptotic death of neuronal cells in the OE peaks at 2 days postbulbectomy, preceding both maximum cell loss and the peak of progenitor cell proliferation by 3–4 days (Holcomb et al., 1995). Since we know that genesis of ORNs proceeds through at least three distinct progenitor cell stages (neuronal CFU,

MASH1⁺ progenitor, and INP), it seems possible that the signal for bulbectomy-induced neurogenesis could act on an early progenitor cell, perhaps even the stem cell: The peak in proliferation at 5–6 days postbulbectomy that is observed would then simply reflect expansion of transit-amplifying cells (MASH1⁺ cells and/or INPs) in response to the early mitogenic stimulus.

We have taken advantage of the purified progenitor/stroma coculture system to test these models in tissue culture. Using the frequency of neuronal colony formation as an assay for neurogenesis, we performed experiments in which purified Rosa26 progenitors were plated onto growth-arrested CD-1 stroma feeder layers in the normal manner, but in half the cultures a large excess (~20-fold) of ORNs was added onto the Rosa26 progenitors 1–2 h after the progenitors were plated (Mumm et al., 1996). In control experiments, a similar fold excess of dissociated CD-1 OE stroma cells was added to Rosa26 progenitor cultures, and cultures were grown for a week, processed for X-gal histochemistry, and then scored for each colony type. The results demonstrated that the addition of ORNs decreases the incidence of neuronal colonies significantly, whereas addition of stroma cells has no significant effect on frequency of neuronal colony formation. Data from typical experiments are illustrated in Figure 10. The frequency of neuronal CFU formation is ~1 in 3600 of plated purified progenitors under normal progenitor/stroma coculture conditions, but when an excess of neurons is added, this frequency drops 2.5-fold,

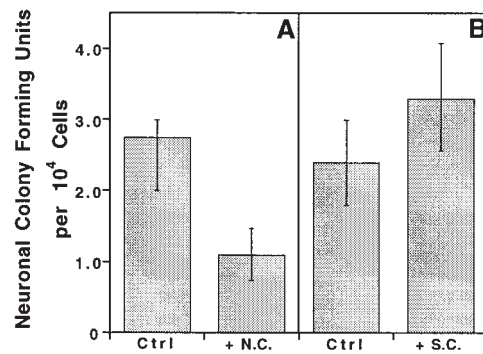


Figure 10 Effect of added neurons and stroma cells on neuronal colony formation. Two experiments in which a large excess of olfactory receptor neurons (A) or stroma (B) cells were added to purified Rosa26 progenitor cultures shortly after plating. The frequency of neuronal colony formation was calculated from the zero-order term of the Poisson equation. Error bars reflect standard deviations resulting from sampling error. (Adapted with permission from Mumm et al., *Proc. Natl. Acad. Sci. USA* 93:11167–11172, © 1996 National Academy of Sciences, U.S.A.)

to ~ 1 in 9,000 [Fig. 10(A)] (Mumm et al., 1996). Two pieces of evidence indicate that this is a specific effect on the development of neuronal colonies: First, no significant effect on the numbers of non-neuronal colony types is seen when excess differentiated ORNs are plated along with purified progenitors (Mumm et al., 1996). Second, no inhibitory effect is observed when an excess of stroma cells is plated with progenitors [Fig. 10(B)], indicating that inhibition cannot be explained as a consequence of added cells simply depleting the medium of nutrients. It is also worth noting that addition of excess ORNs to OE progenitor cell cultures results in a decrease in the number, rather than the size, of neuronal colonies (Mumm et al., 1996). If the signal produced by differentiated ORNs acts upon cells at a very early progenitor stage—for example, neuronal stem cells—causing them to die or to stop producing the downstream neuronal transit amplifying cells that generate ORNs, then this would be the expected outcome.

Thus, although model 2 cannot yet be excluded, the results of these studies are most consistent with model 1 (Fig. 9), since differentiated ORNs appear to produce a signal that inhibits development of neuronal CFUs (Mumm et al., 1996). Interestingly, indirect evidence that the rate of proliferation of cells in the basal OE is regulated by the number of neurons in the epithelium has been provided by several anatomical studies (Mackay-Sim and Patel, 1984; Mackay-Sim et al., 1988; Weiler and Farbman, 1997). A similar regulatory mechanism has been suggested in larval *Xenopus* retina, where ablation of cells *in vivo* by intraocular injection of 6-hydroxydopamine or the excitotoxin kainic acid results in preferential production of new cells of the appropriate type (Reh and Tully, 1986; Reh, 1987).

One other possibility that should be considered is that the presence of excess differentiated ORNs may cause neuronal CFUs to adopt another fate. Such an idea is not without precedent: Shah and colleagues (1994) showed that neural crest stem cells are biased toward a glial, rather than neuronal, fate when grown in glial growth factor (GGF) and that crest-derived neurons express both GGF protein and mRNA, suggesting that neuron-derived GGF affects the fate choice of this type of neural stem cell. In our studies, we noted that addition of excess ORNs to OE progenitor cell cultures resulted (in addition to the decrease in the number of neuronal colonies) in an increase in the number of colonies of spindle-shaped cells (which resemble olfactory Schwann cells morphologically) in two of four experiments, although this result was not statistically significant (Mumm et al., 1996). This finding is

interesting because there is some evidence to suggest that olfactory Schwann cells, the cells that ensheath ORN axons, originate from progenitor cells located within the OE itself (Klein and Graziadei, 1983; Chuah and Au, 1991; Calof and Guevara, 1993). However, we think it unlikely that neuronal colonies and spindle-shaped (putative olfactory Schwann cell) colonies arise from the same cell, as colonies of mixed neuronal and spindle-shaped cells have never been observed in our progenitor cell/stroma cocultures, suggesting that the neuronal CFU may be directed toward an exclusively neuronal (as opposed to glial) fate.

Thus, the results of *in vitro* experiments suggest that differentiated ORNs produce a signal that feeds back to inhibit production of new neurons by their own progenitors, and could explain the surge in neurogenesis in the OE *in vivo* that occurs as a consequence of ORN death following bulbectomy: This increase in neurogenesis could be due to loss of an inhibitory signal normally produced by living ORNs. Uncovering the mechanisms that underlie feedback regulation of neuron production in the OE may be important for understanding the regulation of neuronal production in other parts of the nervous system.

CANDIDATE MOLECULAR REGULATORS OF THE NEURONAL STEM CELL

As discussed above, tissue culture assays of neurogenesis in the OE are useful for testing the behavior of putative OE neuronal stem cells and for determining what signals regulate proliferation and survival of these cells. In particular, there are two important questions that need to be addressed: First, what are the positive factors that allow the OE to continue to generate ORNs on an ongoing basis? Presumably, among such factors will be found those which maintain and/or stimulate proliferation of the neuronal stem cell, as well as other factors that may act locally to stimulate proliferation and differentiation of cells later in the neuronal lineage. Second, what is the identity of the neuron-derived inhibitory signal that acts as a negative regulator of neurogenesis in the OE? In theory, it is this same factor or factors that causes a decrease in the number of neuronal colonies that develop when ORNs are added back in the neuronal colony-forming assay, and this assay should be useful for identifying it. A true understanding of the regulation of neurogenesis in the OE should encompass a molecular identification of both

positive and negative regulatory signals and determination of their mechanisms of action.

Proliferation of OE neuronal progenitor cells is presumably regulated by the activity of mitogenic signals produced in or near the cells on which they act, and so until recently, we were puzzled by the fact that while FGFs, and in particular FGF-2, had been shown by us to have profound stimulatory effects on both INPs and presumed OE neuronal stem cells *in vitro* (DeHamer et al., 1994), there were no convincing data demonstrating expression of a member of the FGF family within the OE itself. However, molecular cloning of the *Fgf-8* gene, together with *in situ* hybridization studies of *Fgf-8* expression patterns in early embryos, has recently provided a possible solution to this puzzle: Reports from several labs indicate that *Fgf-8* mRNA is present in mouse embryos in nasal pits, the primordia of the OE, and that expression is detectable as early as E9 (Crossley and Martin, 1995; Heikinheimo et al., 1994; Mahmood et al., 1995).

These findings prompted us to perform *in situ* hybridization experiments to examine the expression of *Fgf-8* in the OE of later-stage mouse embryos and adult mice. Our initial findings indicate that *Fgf-8* continues to be expressed in the OE at E14—when the rate of neurogenesis is high—and is also present in a small fraction of cells in the basal compartment of adult OE—where neurogenesis is ongoing, albeit at a much lower levels than those observed in the embryo (Calof et al., 1997a; Shou et al., 1998a). Thus, FGF-8 is likely to be produced and available to ORN progenitors undergoing neurogenesis, and may be an endogenous signal that stimulates proliferation of progenitors within the OE, during development and possibly in adults as well. Interestingly, in E14 OE explant cultures *Fgf-8* is expressed in NCAM-negative, non-neurite-bearing migratory cells, at least some of which are capable of incorporating BrDU, suggesting that it is expressed by neuronal progenitor cells (Calof et al., 1997a; Shou et al., 1998a). We have also found that recombinant FGF-8 shares with FGF-2 two major actions on OE neuronal progenitors: It stimulates proliferation of INPs, and it promotes extended neurogenesis in long-term OE explant culture assays to detect stem cell activity in rare explants (DeHamer et al., 1994; Calof et al., 1997a; Shou et al., 1998a). Taken together, these findings suggest that FGF-8 may be an endogenous stimulatory factor that promotes continual neurogenesis in the OE *in vivo*, possibly owing to an effect on OE neuronal stem cells. In addition, the provocative finding that *Fgf-8* appears to be expressed by neuronal progenitors suggests that *Fgf-8* expression may be the basis

of an autocrine loop in the OE, in which neuronal progenitor cells respond to a growth factor that they themselves make.

Equally intriguing is the question of the identity of the neuron-derived inhibitory signal that appears to function to suppress OE neurogenesis *in vitro* (Mumm et al., 1996). Interestingly, in initial experiments to characterize this signal, we have found that ORNs that have been killed by freezing and thawing still retain the ability to inhibit neuronal colony formation, whereas ORNs that have been boiled do not (Calof et al., 1997b). These findings suggest that ORNs inhibit neuronal colony formation not through a complex cell–cell interaction, but more likely through the release of one or more heat-labile factors that they already contain. This result, together with the known growth inhibitory actions of members of the TGF- β superfamily of polypeptide growth factors (Massague and Polyak, 1995) and a recent report demonstrating expression of *Bmp-4* mRNA in the olfactory placode (Wu and Oh, 1996), led us to examine the possibility that BMPs exert effects on OE neurogenesis.

When tested in the colony-forming assay for neuronal stem cell activity (Mumm et al., 1996), BMPs 2 and 4, members of the Decapentaplegic subfamily of TGF- β s, were found to specifically inhibit formation of neuronal colonies, but had no effect on other colony types (Shou et al., 1997, 1998b). Since these two BMPs are highly related structurally and act on the same serine-threonine kinase receptors (Kingsley, 1994; Massague, 1996), we examined the pattern of expression of both factors by *in situ* hybridization in developing and adult mouse OE. These studies demonstrated that *Bmp-4*, but not *Bmp-2*, is expressed in neuronal layers of the OE both during development and in the adult (Shou et al., 1997, 1998b). Taken together, these data suggest that BMP4 expressed by ORNs constitutes at least part of the neuron-derived inhibitory signal detected in our neuronal colony-forming assay. Moreover, experiments varying the time at which BMP-4 is added to colony-forming assays suggest that BMP4's inhibitory action may be exerted primarily on neuronal colony-forming progenitors, the presumptive neuronal stem cells of the OE (Shou et al., 1997).

REMAINING ISSUES AND FUTURE DIRECTIONS

Despite considerable progress, much remains unresolved concerning the identity and developmental regulation of the neuronal stem cell of the OE. Cur-

rently, the neuronal colony-forming cell (Mumm et al., 1996) is our best candidate for the stem cell, but several questions need to be answered before this can be regarded as conclusive. We have no molecular marker that distinguishes this cell from other progenitor cells in the OE, so we are unable to follow its development specifically, *in vitro* or *in vivo*. Owing to this absence of a stem cell-specific marker, we have been limited in our study of the stem cell by assays of the developmental potential of the progenitor cell population *in toto*, and these studies still have gaps. For example, we do not yet know the extent of the neuronal CFU's capacity for self-renewal (this has only been tested up to 2 weeks in culture), and it is likely that our present culture methods will need to be improved before such experiments can be performed. Thus, markers specific for the OE neuronal stem cell and improved culture conditions for analyzing its behavior remain an important goal.

Considering these issues, it is interesting that one way in which the neuronal CFU resembles other stem cells is in its apparent requirement for factors provided by cells derived from supporting stroma (Mumm et al., 1996). For example, stromal cells play a crucial role in regulating survival of stem cells in hematopoietic development (Deryugina et al., 1993) and in the development of epidermal stem cells *in vitro* (Jones and Watt, 1993). Identification of the stroma-derived factor(s) that are crucial for neuronal CFU development should provide important information about the molecular regulation of neuron production in the OE and may also provide clues as to the identity of the factors that permit lifelong neuron renewal in this tissue.

Finally, much remains to be done concerning the mechanisms of action of stimulatory and inhibitory factors affecting the neuronal CFU and other neuronal progenitor cells in the OE. Which members of the BMP and FGF families have effects on OE neurogenesis? When are these factors expressed during development and surgery-induced neurogenesis? How do BMPs and FGFs regulate stem cell behavior? Do they affect cell death, cell fate, or differentiation of these cells? What other growth factors play a role in these processes? The answers to these questions will have important implications for our understanding of the molecular basis of growth control in the nervous system.

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