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Factors Regulating Neurogenesis and Programmed Cell Death in Mouse Olfactory Epithelium[a]

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ABSTRACT: To identify factors regulating neurogenesis and programmed cell death in mouse olfactory epithelium (OE), and to determine the mechanisms by which these factors act, we have studied mouse OE using two major experimental paradigms: tissue culture of embryonic OE and cell types isolated from it; and ablation of the olfactory bulb ('bulbectomy') of adult mice, a procedure that induces programmed cell death of olfactory receptor neurons (ORNS) and a subsequent surge of neurogenesis in the OE *in vivo*. Such experiments have been used to characterize the cellular stages in the ORN lineage, leading to the realization that there are at least two distinct stages of proliferating neuronal progenitor cells interposed between the ORN and the stem cell that ultimately gives rise to it. The identification of a number of different factors that act to regulate proliferation and survival of ORNs and progenitor cells suggests that these multiple cell stages may each serve as a control point at which neuron number in the OE is regulated. Our recent studies of neuronal colony-forming progenitors (putative stem cells) of the OE suggest that even these cells, at the earliest stage in the ORN lineage so far identified, are subject to such regulation: if colony-forming progenitors are cultured in the presence of a large excess of differentiated ORNs, then the production of new neurons by progenitors is dramatically inhibited.[6] This result suggests that differentiated ORNs produce a signal that feeds back to inhibit neurogenesis by their own progenitors, and provides a possible explanation for the observation that ORN death, consequent to bulbectomy, results in increased neurogenesis in the OE *in vivo:* death of ORNs may release neuronal progenitor cells from this inhibitory signal, produced by the differentiated ORNs that lie near them in the OE. Our current experiments are directed toward identifying the molecular basis of this inhibitory signal, and the cellular mechanism(s) by which it acts.

Understanding the basic biology of neuron production is of fundamental importance for understanding how the final form and function of the nervous system are achieved. Production of most neurons takes place during embryogenesis, gradually slowing and in most cases permanently ceasing toward the end of development.[1,2] How does the developing nervous system know when to *stop* producing neurons? Signals that halt neuron production at the correct times and in the correct locations must exist to ensure that

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the proper form of the nervous system is attained, but persistence of these same signals may also account for the fact that neurons cannot be regenerated when their numbers have been reduced by developmental defects, injury, or aging.

To identify the molecules that regulate neurogenesis and related processes during nervous system development, we have concentrated on developing a system in which behavior of neuronal progenitor cells can be observed and manipulated readily: the olfactory epithelium (OE) of the mouse. The OE is morphologically and functionally similar to the embryonic neuroepithelia that generate the rest of the nervous system, but is much simpler, in that it produces large numbers of a single type of neuron, the olfactory receptor neuron (ORN). We have taken advantage of this simplicity to study mouse OE using both *in vitro* and *in vivo* experimental approaches. These include tissue culture of embryonic OE and cell types isolated from it,[3–6] and ablation of the olfactory bulb ('bulbectomy') of adult mice, a procedure that induces programmed cell death of ORNs and a subsequent surge of neurogenesis in the OE *in vivo*.[5,7,8] Such studies have been used to characterize the cellular stages in the ORN lineage, leading to the realization that there are at least two distinct stages of neuronal transit amplifying progenitor cells interposed between the ORN and the stem cell that ultimately gives rise to it: Expression of the transcription factor mammalian achaete-scute homologue-1 (MASH1) marks cells at the earlier of these stages,[7] whereas the progenitor cells that give rise directly to ORNs, the immediate neuronal precursors (INPs), are MASH1-negative.[3,7] In addition, such studies have led to the realization that members of the fibroblast growth factor (FGF) family act as stimulatory factors regulating neurogenesis in the OE; notably, FGFs act to stimulate cell divisions of ORN progenitors, particularly INPs.[4,9]

The OE forms during embryonic development, yet—unlike nearly all other regions in the vertebrate nervous system—can continue to generate neurons throughout adulthood.[10] Because of this, it has been possible to learn that production of neurons in the OE is a regulated process that serves to maintain the number of ORNs at a particular level. Thus, in normal animals, in which ORNs are constantly dying in low numbers (due 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 upregulated just until the original state of the OE is restored. In one such manipulation, when one olfactory bulb is removed from the brain of an adult rodent, nearly all ORNs in the OE on the side that innervated that bulb undergo apoptotic cell death[5]. As ORNs die and the OE degenerates (decreases in thickness), neuronal progenitor cells, which lie underneath the dying neurons within the OE, increase their proliferation and replace the lost ORNs.[7,8]

These properties of the OE suggest that somehow, neuronal progenitor cells 'read' the number of differentiated neurons in their immediate environment, and regulate the production of new neurons accordingly. Recently, our studies to investigate the characteristics of OE neuronal progenitors in tissue culture have led us to the discovery of what we believe to be the *in vitro* correlate of this phenomenon: In studies to identify conditions that support survival of neuronal stem cells from the OE, we purified neuronal progenitor cells from embryonic OE using selective dissociation and immunological panning techniques.[6] When grown under appropriate conditions, a small fraction of these purified progenitors (about 1 in 3600) gives rise to colonies of proliferating cells that continue to divide and generate postmitotic ORNs for at least 2 weeks in culture.[6] Our current hypothesis is that the cells that give rise to neuronal colonies are the rare, self-renewing stem cells that must exist in order for the OE to have the capacity for continuous neuron production, and so we have named them *neuronal colony-forming*

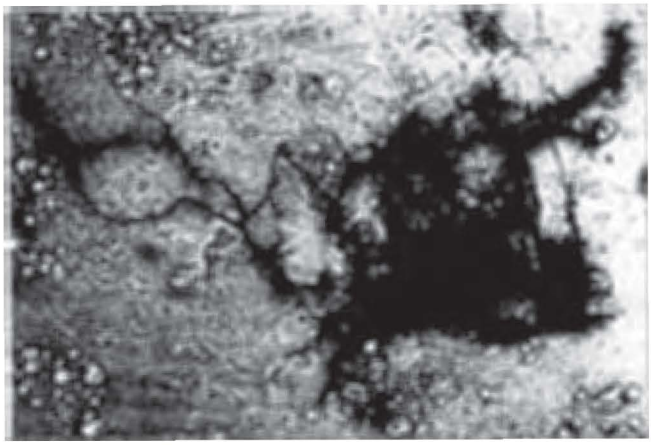

FIGURE 1. Neuronal colony (neuronal CFU) at 7 days *in vitro.* Colony has been stained with antibody to neural cell adhesion molecule (N-CAM, a marker for all postmitotic ORNs[3,4]), detected with horseradish peroxidase (HRP)-conjugated secondary antibody [cf. Ref. 6].

TABLE 1. Effect of Added ORNs on the Development of Neuronal Colonies

A. Differentiated ORNs inhibit the development of neuronal colonies

	Control	ORNs Added	% Change	
Neuronal colonies				
Exp. 1	23	10	−56.5	
Exp. 2	12	6	−50.0	
Exp. 3	16	6	−62.5	
Exp. 4	15	10	−33.3	
Mean	16.5	8	−50.6	$p < 0.02$
All other colonies				
Exp. 1	107	123	+15.0	
Exp. 2	37	31	−16.2	
Exp. 3	42	60	+42.9	
Exp. 4	135	113	−16.3	
Mean	80.25	81.75	+6.35	$p = 0.96$

B. Preliminary characterization of the ORN inhibitory signal

Condition	Control	Experimental	% change
Neuronal colonies			
Living ORNs added	12	6	−50.0
Living stroma cells added	15	20	+33.3
Freeze-thawed ORNs added	23	10	−56.5
Boiled ORNs added	11	11	0.0

NOTE: For comparison, colony numbers for different experiments have been normalized to 96,000 input cells, the number normally plated in a 96-well plate in each test condition. (Data in A adapted from Ref 6.)

cells (CFUs, colony-forming units), by analogy to CFUs thought to be indicative of stem cells in the hematopoietic lineage. An example of a neuronal colony is shown in FIGURE 1.

Interestingly, when purified progenitors are grown in the presence of a 20-fold excess of differentiated ORNs, development of neuronal colonies is *inhibited* by two-fold or more (TABLE 1A, adapted from Ref. 6). This inhibitory effect of ORNs on neuronal CFU development appears to be specific: Not only are other colony types that arise in these cultures not affected by addition of ORNs (TABLE 1A), but also a similar-fold excess of nonneuronal cells added to progenitor cell cultures has no effect on the development of neuronal colonies (cf. Ref. 6). These results suggest that differentiated ORNs produce a signal that feeds back to inhibit neurogenesis by their own progenitors, and provides a possible explanation for the observation that ORN death, consequent to bulbectomy, results in increased neurogenesis in the OE *in vivo:* death of ORNs may release neuronal progenitor cells from this inhibitory signal, produced by the differentiated ORNs which lie above them in the OE. A preliminary biochemical characterization, shown in TABLE 1B, suggests that a heat-labile macromolecule(s) is responsible for ORN-mediated feedback inhibition of neurogenesis, and our current experiments are directed toward identifying this factor and the cellular mechanism(s) by which it acts.[a]*Supported by NIH Grants DC02180 and NS32174 to A.L.C.*

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