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Genesis of Olfactory Receptor Neurons In Vitro: Regulation of Progenitor Cell Divisions by Fibroblast Growth Factors

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Summary

Olfactory receptor neurons are produced continuously in mammalian olfactory epithelium in vivo, but in explant cultures neurogenesis ceases abruptly. We show that in vitro neurogenesis is prolonged by fibroblast growth factors (FGFs), which act in two ways. FGFs increase the likelihood that immediate neuronal precursors (INPs) divide twice, rather than once, before generating neurons; this action requires exposure of INPs to FGFs by early G1. FGFs also cause a distinct subpopulation of explants to generate large numbers of neurons continually for at least several days. The data suggest that FGFs delay differentiation of a committed neuronal transit amplifying cell (the INP) and support proliferation or survival of a rare cell, possibly a stem cell, that acts as a progenitor to INPs.

Introduction

The mammalian olfactory epithelium (OE) is uniquely suited to studies of how neurogenesis is controlled. In the OE, proliferation of neuronal precursor cells and differentiation of their progeny into olfactory receptor neurons (ORNs) begin during embryogenesis and continue throughout life (Graziadei and Monti Graziadei, 1978). Even in adults, these processes appear to be regulated, since in vivo manipulations that lead to death of ORNs result in increased mitotic activity of neuronal precursors (Monti Graziadei and Graziadei, 1979; Schwartz Levey et al., 1991).

We previously developed an explant culture system for purified embryonic mouse OE to study neurogenesis under defined conditions. Three major cell types are present in these cultures: basal cells, which express keratins; postmitotic ORNs, which express the neural cell adhesion molecule N-CAM; and a third cell type, which expresses neither keratins nor N-CAM (Calof and Chikaraishi, 1989). The keratin$^-$, N-CAM$^-$ cell is a migratory cell that rapidly sorts out from the basal cells (which remain in the body of the explant), synthesizes DNA, and divides as it migrates and is actually the direct precursor of N-CAM$^+$ ORNs. We call this cell the immediate neuronal precursor (INP; Calof and Chikaraishi, 1989; Calof et al., 1991). Indirect evidence that INPs exist in vivo and give rise directly to ORNs has been obtained by others (Mackay-Sim and Kittel, 1991). INPs may in fact be the so-called "globose" basal cells of the OE (Graziadei and Monti Graziadei, 1979), although markers are not yet available with which to test this idea.

Although explant cultures of OE made it possible to observe neurogenesis in vitro, it was found that ORN production ceased after 1–2 days in culture. Specifically, INPs divided only once, nearly all their progeny became ORNs, and new INPs did not appear (Calof and Chikaraishi, 1989). Since these studies were carried out in defined, serum-free medium, it seemed that it might be possible to restore continual neurogenesis (as occurs in OE in vivo) by addition of appropriate polypeptide growth factors. Several classes of growth factors and their receptors are highly expressed in or near the early nervous system and are suspected of regulating proliferation and/or differentiation of neuronal precursors (e.g., Klein et al., 1989; Heuer et al., 1990; Maisonpierre et al., 1990; Reid et al., 1990; Yeh et al., 1991; Schecterson and Bothwell, 1992). Indeed, in vitro studies indicate that polypeptide growth factors and as yet uncharacterized factors affect both the process of neurogenesis and the fates of neural precursor cells (e.g., Gao et al., 1991; Temple and Davis, 1994; Shah et al., 1994).

This manuscript deals with mechanisms by which a major class of polypeptide growth factors, fibroblast growth factors (FGFs), regulates production of ORNs in culture. Several members of the FGF family (FGF1, FGF2, FGF4, and FGF7) were found to prolong significantly the proliferation of ORN progenitor cells, whereas growth factors from several other families did not. Moreover, expression of tyrosine kinase FGF receptors (FGFRs) FGFR1 and FGFR2 was detected in a purified cell fraction consisting solely of INPs and ORNs. Analysis of FGF action indicates that FGFs affect olfactory neurogenesis in at least two ways: first, FGFs enable INPs to undergo two rounds, rather than one round, of division before differentiating into ORNs, and second, FGFs appear to permit progenitor cells that are present in only a small fraction of OE explants to proliferate for several more days before generating ORNs. The first effect of FGFs suggests that the bulk of INPs in OE cultures are transit amplifying cells, i.e., cells committed to both a limited number of divisions and a specific (neuronal) fate. The second effect of FGFs suggests the possible existence of neuronal stem cells in some, but not all, OE explants.

Results

FGFs Promote Late-Occurring Proliferation in OE Explant Cultures

To identify polypeptide growth factors that could prolong OE neurogenesis in vitro, a two-step approach was taken: first, to identify growth factors that in-

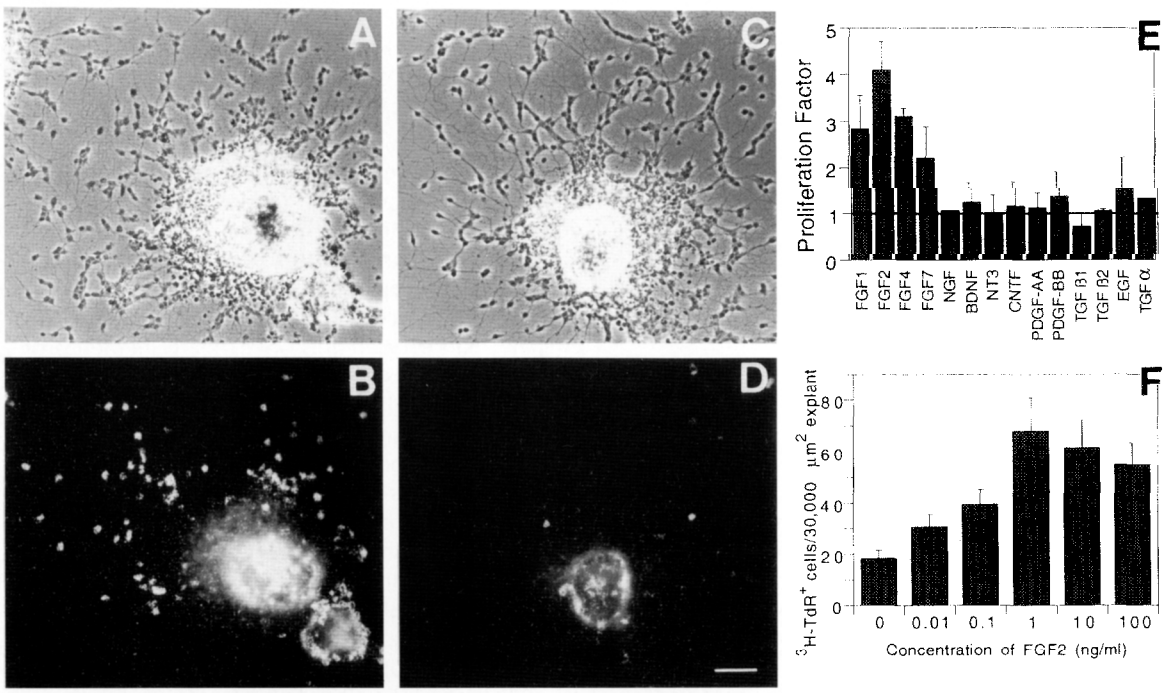

Figure 1. Results of Growth Factor Screening Assays: FGFs Promote Late-Occurring Proliferation in OE Cultures

(A–D) Explant cultures, with indicated growth factors continuously present, were incubated with ^{3}H-TdR (0.1 μCi/ml) from t = 24 to 48 hr in culture, fixed, and processed for autoradiography. (A) Phase-contrast, FGF2-treated culture (10 ng/ml). (B) Same field as (A), dark field. (C) Phase-contrast, control culture (no growth factor). (D) Same field as (C), dark field. Bar, 50 μm.

(E) Growth factor screening assays. Bars represent mean proliferation factor ± SD for growth factors at optimum tested concentrations (FGF1, 100 ng/ml; FGF2, 1 ng/ml; FGF4, 10 ng/ml; FGF7, 10 ng/ml; NGF, BDNF, and NT-3, 50 ng/ml; CNTF, PDGF-AA, PDGF-BB, TGFβ1 and TGFβ2, 10 ng/ml; EGF and TGFα, 20 ng/ml. FGF4 and FGF7 were each also tested at 1 and 100 ng/ml and gave effects similar to those observed at 10 ng/ml [data not shown]). Proliferation factors were calculated as follows: for each explant, the explant labeling index was calculated as the number of migratory cells with silver grains over their nuclei divided by the area of the explant (measured using NIH Image 1.52). The proliferation factor is the ratio of the mean explant labeling index in a given condition to that of controls (no added growth factor) grown on the same day. Percent of error for these ratios (the square root of the sum of the squares of percent errors [from SEM] of the two labeling indices being compared) averaged ~20%. ANOVA followed by Dunnett's test (for multiple comparisons against a single control; Glantz, 1992) was performed for every experiment. No growth factors other than the FGFs were found to have statistically significant effects ($p < .05$) on the explant labeling index.

(F) Dose-response analysis for FGF2. Points represent mean ± SEM for explant labeling index at each concentration. The unit of area used in normalizing the explant labeling indices was 30,000 μm^2, the approximate mean explant area for all tests.

creased "late" proliferation among cells likely to be neuronal precursors, and second, to show that late-proliferating cells indeed give rise to ORNs. For the first step, OE explants were cultured in serum-free medium supplemented with 1 of several growth factors for 24 hr (a time by which, in the absence of added growth factors, neuronal precursor proliferation has largely ceased [Calof and Chikaraishi, 1989]). The growth factor was then replenished, and a low level (0.1 μCi/ml) of ^{3}H-thymidine (^{3}H-TdR) was added. After a further 24 hr, cultures were fixed and processed for autoradiography.

Growth factors that were tested represented several families known to affect proliferation of glial and neuronal progenitors, e.g., platelet-derived growth factors, neurotrophins, FGFs, and ciliary neurotrophic factor (CNTF; see Discussion). In addition, some factors known to affect epithelial or neuroepithelial proliferation and differentiation, or previously observed by us to affect proliferation of cells in OE cultures (e.g., epidermal growth factor [EGF] and transforming growth factor βs [TGFβs]), were also tested (Calof and Chikaraishi, 1989; Calof et al., 1991; Anchan et al., 1991; Mahanthappa and Schwarting, 1993). An example is shown in Figures 1A–1D. Substantially more ^{3}H-TdR$^+$ cells are observed in and around a typical explant grown in FGF2 (A and B) than in and around a comparable explant grown without any added growth factor (C and D). Interestingly, in cultures grown in FGF2, ^{3}H-TdR-incorporating migratory cells often appear in patches of 4 or more labeled cells (B).

Growth factor effects on neuronal precursor proliferation were quantified by examining only the migratory cells in OE explant cultures, i.e., cells that leave the body of the explant and disperse onto the culture substratum. Previous data show that virtually all cells in the migratory fraction are either neurons (ORNs) or neuronal precursors (INPs), whereas the explants themselves also contain other proliferating cell types, primarily keratin$^+$ basal cells (Calof and Chikaraishi,

Table 1. FGF2 Promotes Late-Occurring Proliferation of Migratory Cells but Does Not Affect Their Migration

Condition	A. ^{3}H-TdR Labeling Index	B. ^{3}H-TdR$^+$ Cells per 30,000 μm^2 Explant (mean ± SEM)	C. Migratory Cells per 30,000 μm^2 Explant (mean ± SEM)	D. Corona Area/Explant Area (mean ± SEM)	E. Labeling Index of Dissociated INPs + ORNs (mean ± SEM)
FGF2 10 ng/ml	11.13% (n = 4680) PF = 3.14	63.93 ± 18.21 PF = 3.26	560 ± 62	5.22 ± 0.51	3.05 + 0.19% PF = 6.93
No growth factor	3.54% (n = 4977)	19.59 ± 3.93	505 ± 49	5.59 ± 0.62	0.44 ± 0.27%
NGF 50 ng/ml	3.17% (n = 4640) PF = 0.90	14.52 ± 2.88 PF = 0.74	467 ± 38	4.91 ± 0.25	ND

(A–D) Explants were analyzed from growth factor screening assays illustrated in Figure 1. (A) Percentage of total migratory cells incorporating ^{3}H-TdR. n, number of cells counted; PF, proliferation factor, the experimental labeling index divided by labeling index in no growth factor. (B) ^{3}H-TdR labeling index normalized to explant area, calculated as described in Figure 1E. The unit of area used in normalizing the data was 30,000 μm^2, the approximate mean explant area in all conditions. Thus, (B) reflects the number of migratory ^{3}H-TdR$^+$ cells surrounding an average-sized explant. (C) Total number of migratory cells per explant (normalized to average explant area). (D) Ratio of area covered by an explant's migratory cells to area of the explant itself. Labeling index normalized to explant area in FGF2 (B) was significantly different ($p < .05$) from negative control; labeling index in NGF was not (ANOVA and Dunnett's tests for multiple comparisons against a single control; Glantz, 1992). Neither FGF2 nor NGF showed a significant difference from negative control in the ratios of migratory cells to explant area (C) or area of migration to explant area (D). The apparent paradox that FGF2, a factor which promotes proliferation of migratory cells, does not increase significantly the average total number of migratory cells per explant (C) is in part accounted for by the fact that proliferating INPs constitute only 25%–30% of migratory cells (the rest are postmitotic ORNs; Calof and Lander, 1991). In addition, as later data demonstrate (Figure 5), the observed effect of FGF2 on explant labeling indices requires increased proliferation of only 12%–30% of INPs. Thus, the expected maximum increase in total number of migratory cells in FGF2 is only about 9%. The data in fact show such an increase (C), but the increase was not statistically significant.
(E) BrdU labeling index of the dissociated neuronal fraction, cultured for 48 hr, with BrdU present from 24 to 48 hr in culture. The percentage of BrDU$^+$ cells was calculated for ten fields in each of triplicate cultures; over 9000 total cells were counted for each condition.

1989; Calof and Lander, 1991). Limiting analysis to migratory cells was also considered justifiable because available data do not suggest that substantial differences exist between INPs and ORNs that do not migrate (some remain on top of or associated with the edges of explants) and those that do. For example, all or most ORNs and INPs seem to have the ability to migrate, and the proportion of ORNs to INPs is about the same in the migratory cells as in OE cultures as a whole (Calof and Chikaraishi, 1989; Calof and Lander, 1991). Additionally, the dispersal of migratory cells enables their numbers to be counted and their phenotypes to be assessed accurately.

Indices of ^{3}H-TdR labeling for migratory cells (also referred to below as "neuronal cells" or "neuronal cell fraction") initially were calculated as follows: for each explant, the number of ^{3}H-TdR$^+$ cells surrounding it was normalized to explant area (to control for variation in explant size), and data from many explants were averaged to yield a mean explant labeling index. The ratio of the mean explant labeling index for a given growth factor to the mean explant labeling index observed for parallel cultures grown in the absence of added growth factor was then calculated. This ratio (proliferation factor) depicts the fold increase in ^{3}H-TdR labeling caused by the growth factor tested. Thus, a proliferation factor of 1 indicates that a growth factor had no detectable effect.

Figure 1E summarizes these results for 14 different growth factors tested. All 4 tested members of the FGF family consistently produced 2- to 4-fold increases in labeling indices that were statistically significant (see legend). In contrast, no other tested growth factor had a significant effect. Because FGF2 showed the greatest effect, this factor was used in subsequent experiments to explore the mechanism of action of members of the FGF family. A dose-response analysis of FGF2's effect on the labeling index (Figure 1F) showed a peak response at 1 ng/ml and EC_{50} of ~0.1 ng/ml (~6 pM), on the order of what has been observed in other systems (e.g., Olwin and Haushka, 1986).

To prove definitively that the effects of FGF2 reflect a real increase in late-occurring proliferation, it was necessary to rule out certain artifacts. For example, exclusion of nonmigratory cells from the analysis, or normalization of explant labeling indices to explant area, could have biased the data if FGF2 had substantial effects on cell migration or explant spreading (which influences explant area). To address these issues, cultures grown in FGF2 were compared with two types of negative control cultures: no added growth factor and nerve growth factor (NGF). In Table 1, column A shows the true labeling indices for migratory cells, i.e., the fraction of total migratory cells that was ^{3}H-TdR$^+$, in each condition; column B contains labeling indices normalized to explant area, as in Figure 1E. The fold increase in proliferation (proliferation factor) observed in FGF2 was essentially identical regardless of which method of analysis was used (3.14 versus 3.26). Thus, FGF2's effects on proliferation are independent of explant area, and explant labeling indices apparently provide a reasonable measure of proliferation. Column C shows that FGF2 does not alter significantly the number of cells that migrate from each explant or the area covered by migratory cells surrounding each explant.

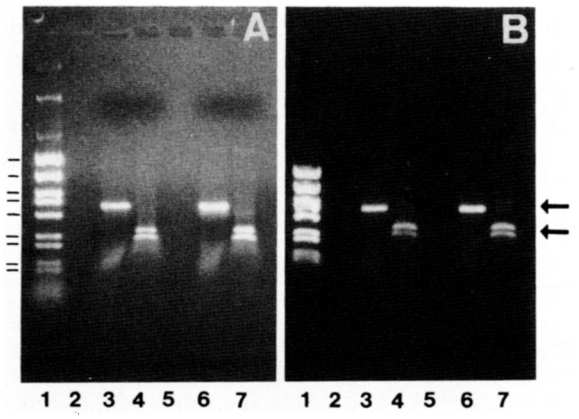

Figure 2. Analysis of FGF Receptor Expression in Purified OE

Total RNA and cDNA were prepared from purified OE (A) or the isolated neuronal fraction (INPs + ORNs) of OE suspension cultures (B), and PCR amplification was performed using FGF receptor-specific (FR) primers (see Experimental Procedures).

(A; lane 1) Molecular weight markers, in descending order by size (indicated by black dashes on left): 1075, 796, 591, 517, 396, 255, 205, 112, and 102 bp. (2) FR1 primers, no-RT control (30 cycles). (3) FR1 primers + OE cDNA (30 cycles; expected size, 461 bp; top arrow). (4) FR1 primers plus OE cDNA (30 cycles), BglI digest (expected sizes, 244 and 217 bp; bottom arrow). (5) FR2 primers, no-RT control (27 cycles). (6) FR2 primers plus OE cDNA (27 cycles; expected size, 452 bp; top arrow). (7) FR2 primers plus OE cDNA (27 cycles), MluI digest (expected sizes, 252 and 200 bp; bottom arrow).

(B) Lanes are the same as in (A), except that cDNA was from dissociated INPs + ORNs, and PCR reactions were run for 35 cycles.

Table 2. Pulse Labeling Indices of Migratory Neuronal Cells Are Unaffected by FGF2 at Early Times in Culture

	Percentage of Migratory Cells That Are ^{3}H-TdR$^+$	
Time in Culture	FGF2 (10 ng/ml)	No Growth Factor
6 hr	19.01 ± 0.20	16.32 ± 2.58
10.5 hr	9.62 ± 0.90	11.43 ± 0.47
12 hr	9.67 ± 2.14	9.80 ± 0.57

Explant cultures were fixed at the indicated times, with ^{3}H-TdR (5 μCi/ml) present in the culture medium for the last 2 hr in culture. All migratory cells surrounding an explant were counted for 10 explants in each culture, with a total of >1500 cells counted in each culture. Values shown are mean ± SEM for explants grown on the same day. Labeling indices for cultures grown in FGF2 versus no growth factor did not differ significantly from each other at any of the three timepoints tested ($p > .2$ in all cases by Student's t test; cf. Glantz, 1992).

Because OE explant cultures contain cell types other than migratory neuronal cells (primarily basal cells, which do not migrate; cf. Calof and Chikaraishi, 1989), it was possible that the effects of FGFs might be exerted indirectly, e.g., through basal cells, which might then provide signals affecting the proliferation of migratory neuronal cells. To examine this possibility, a previously described technique was used to isolate dissociated INPs and ORNs (the neuronal cell fraction) free from basal cells (Calof and Lander, 1991; Calof et al., 1994a). These dissociated cells were cultured in the presence or absence of FGF2 under the same conditions as those used in Figure 1 (except that bromodeoxyuridine [BrdU] was substituted for ^{3}H-TdR). As Table 1, column E shows, FGF2 markedly increased proliferation in this isolated neuronal fraction. In fact, the effect of FGF2 on the labeling index (a nearly 7-fold increase) was even higher than that seen in explant cultures (Figure 1E and Table 1; see Discussion).

Together, the data indicate that the apparent stimulatory effect of FGFs on late proliferation is real and reflects a *direct* action of FGF2 on the neuronal cell fraction. Since the only proliferative cells in this fraction are the N-CAM$^-$ INPs, the results strongly suggest that FGF2 acts directly on these precursors of ORNs.

FGFRs Are Expressed by Neuronal Cells of the OE

Most effects of FGFs are thought to be mediated through FGFRs. To confirm that such receptors are expressed in OE and to identify which ones are present, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed using primers specific for the transmembrane domains of FGFR1, FGFR2, FGFR3, and FGFR4 (Figure 2). When RT-PCR was performed using RNA prepared from either purifed OE (Figure 2A) or isolated INPs plus ORNs (Figure 2B), amplification products of the expected size (~450 bp) were obtained with primer sets specific for FGFR1 and FGFR2. Identity of these amplification products with appropriate regions of the FGFR1 and FGFR2 cDNAs (Lee et al., 1989; Dell and Williams, 1992) was confirmed by sequencing. In contrast, we failed to amplify transcripts for FGFR3 and FGFR4 from OE RNA or RNA from the isolated neuronal cell fraction, despite the ability of the primers to amplify correctly sized products from E11 mouse head RNA (data not shown). We conclude that transcripts for FGFR3 and FGFR4 either are not present in embryonic mouse OE or are below the limits of detection.

Expression of FGFR1 and FGFR2 by neuronal cells of the OE is consistent with the observed proliferative effects of FGFs on these cells. Although the PCR primers used in these experiments do not allow us to distinguish among splice variants of FGFR1 and FGFR2 (Givol and Yayon, 1992), all 4 FGFs (FGFR1, FGFR2, FGFR4, and FGFR7) tested in this study have been shown to bind with high affinity to at least 1 variant of FGFR1 or FGFR2 (Dionne et al., 1990; Mansukhani et al., 1990; Rubin et al., 1989).

FGF Stimulates ORN Precursors to Undergo Multiple Divisions before Giving Rise to Neurons

The simplest explanation for the direct effect of FGFs on late proliferation by neuronal cells in OE cultures is that these growth factors act as mitogens. For example, FGFs might cause some cells that had previously been "resting" to enter the cell cycle. Alternatively, FGFs might cause cycling cells to proliferate faster, by decreasing the length of the cell cycle. Both types of mitogenic effect would be expected to cause an

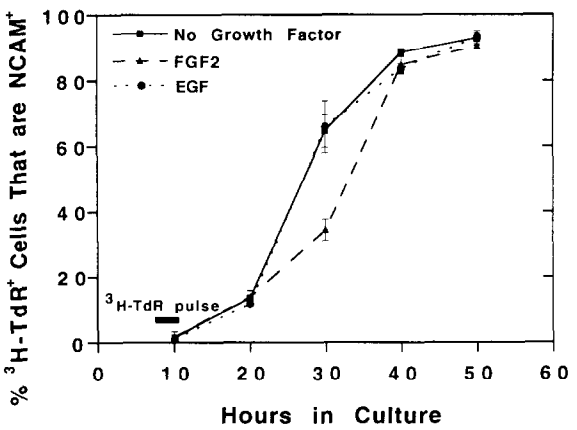

Figure 3. FGF2 Causes a Delay in Acquisition of N-CAM Immunoreactivity by Olfactory Neuron Precursors

Explant cultures were grown continuously in FGF2 (10 ng/ml), EGF (50 ng/ml), or no added growth factors. After 8 hr, cultures were pulsed with ^{3}H-TdR (5 μCi/ml) for 2 hr, then either fixed or re-fed with medium containing 50 μM unlabeled TdR ("chase"). At 10 hr intervals, cultures were fixed and processed for N-CAM immunocytochemistry and autoradiography. Cultures were analyzed at 6300× magnification using phase, epifluorescence, and bright field optics. At least 200 ^{3}H-TdR$^+$ cells in a minimum of ten fields were scored in each culture. Data points are mean ± range from duplicate cultures.

increase in labeling index in an experiment in which cells are exposed for 2 hr to ^{3}H-TdR and then fixed. This experiment was performed on OE explants cultured in FGF2 or no growth factor for various times (up to 12 hr). The results (Table 2) revealed no significant effect of FGF2 on labeling index.

These data provided an early hint that the effect of FGFs might not be on the entry of cells into the cell cycle, nor on cell cycle kinetics, but rather on the number of cycles that cells undergo before terminally differentiating. A stronger indication that this is the case came from analysis of a pulse-chase study designed to follow the fate of proliferating cells in OE cultures in the presence or absence of FGF2.

In this experiment (Figure 3), OE explant cultures—grown in no growth factor, FGF2, or a control growth factor (EGF)—were pulsed with ^{3}H-TdR from 8 to 10 hr in vitro and chased with unlabeled TdR to various time points. The cultures were fixed and analyzed for immunoreactivity to N-CAM (a neuron-specific marker in the OE) in ^{3}H-TdR-labeled cells. As shown in a previous study (Calof and Chikaraishi, 1989), the migratory cells that incorporate ^{3}H-TdR are initially all N-CAM$^-$. In the present study, this was found to be true regardless of the presence or absence of growth factors (Figure 3). By 50 hr in culture, more than 90% of ^{3}H-TdR$^+$ cells in all conditions acquired N-CAM immunoreactivity. Thus, the great majority of proliferating, N-CAM$^-$ migratory cells gave rise to N-CAM$^+$ ORNs. The fact that virtually all did so in the presence as well as the absence of FGF2 implies that FGF2 did not divert INPs from the neuronal fate to which they appear to be committed.

Interestingly, however, Figure 3 reveals a marked effect of FGF2 on the rate at which neuronal fate was attained. Compared with untreated or EGF-treated cultures, FGF2-treated cultures showed a distinct lag in N-CAM acquisition by ^{3}H-TdR-labeled cells: at 30 hr in culture, only half as many such cells had begun to express N-CAM in the presence of FGF2 as in control cultures. This apparent delay in neuronal differentiation could be explained most easily if the primary effect of FGF2 were to cause some neuronal precursors (INPs) to undergo additional divisions before terminally differentiating. This effect would also explain why FGFs increase the number of cells that proliferate at late times in culture (Figure 1), without significantly altering the pulse-labeling index at early times in culture (Table 2).

To test this hypothesis, an experiment was performed to detect and quantitate ORNs that were generated as a result of two successive rounds of cell division in culture. This was done (Figure 4) by sequentially labeling OE explant cultures with two S-phase markers, BrdU and ^{3}H-TdR, administered far enough apart in time (≥12 hr) that any double-labeled neuron would have had to be the progeny of a cell that passed through two successive S phases in culture. (That 12 hr was a sufficiently long interval is demonstrated in the experiment described in Figure 5, below.) The results, shown in Figure 4, indicate that FGF2 causes a large and significant increase in the number of ORNs generated by two, rather than one, precursor cell divisions. In (A)–(C), examples are shown of cultures grown in FGF2, in which at least 3 N-CAM$^+$ ORNs have incorporated both BrdU and ^{3}H-TdR in their nuclei (arrows). Figure 4D shows that the incidence of N-CAM$^+$, BrdU-labeled neurons also labeled by ^{3}H-TdR is 4- to 5-fold greater in FGF2-treated cultures than in controls. Notably, this increase is similar in magnitude to the 3- to 5-fold effect of FGF2 on overall "late" proliferation that was observed in Figure 1E.

To characterize the time course of the second round of precursor division that occurs in FGF, cultures grown in FGF2 were given an early, brief pulse of BrdU (4–6 hr in culture). The BrdU pulse was either overlapped with a 2 hr pulse of ^{3}H-TdR or followed by a 2 hr ^{3}H-TdR pulse at a later time. The results are shown in Figure 5A. Initially, when the pulses of BrdU and ^{3}H-TdR overlap, virtually 100% of the BrdU$^+$ cells are double-labeled, as expected for a cohort of cells labeled simultaneously with two different S-phase markers. The percentage of double-labeled cells drops rapidly as the BrdU-labeled cells exit S phase and progress through G2, M, and G1 and then increases again to a second peak ~17 hr later, as some cells progress into a second S phase and again become able to incorporate ^{3}H-TdR. In this experiment, ~12% of INPs that could be labeled in a first S phase also underwent a second round of division in FGF.

These data allow an estimation of the INP cell cycle to be made and fit well with a model predicting a total cell cycle length of ~17 hr, with an S phase of ~8

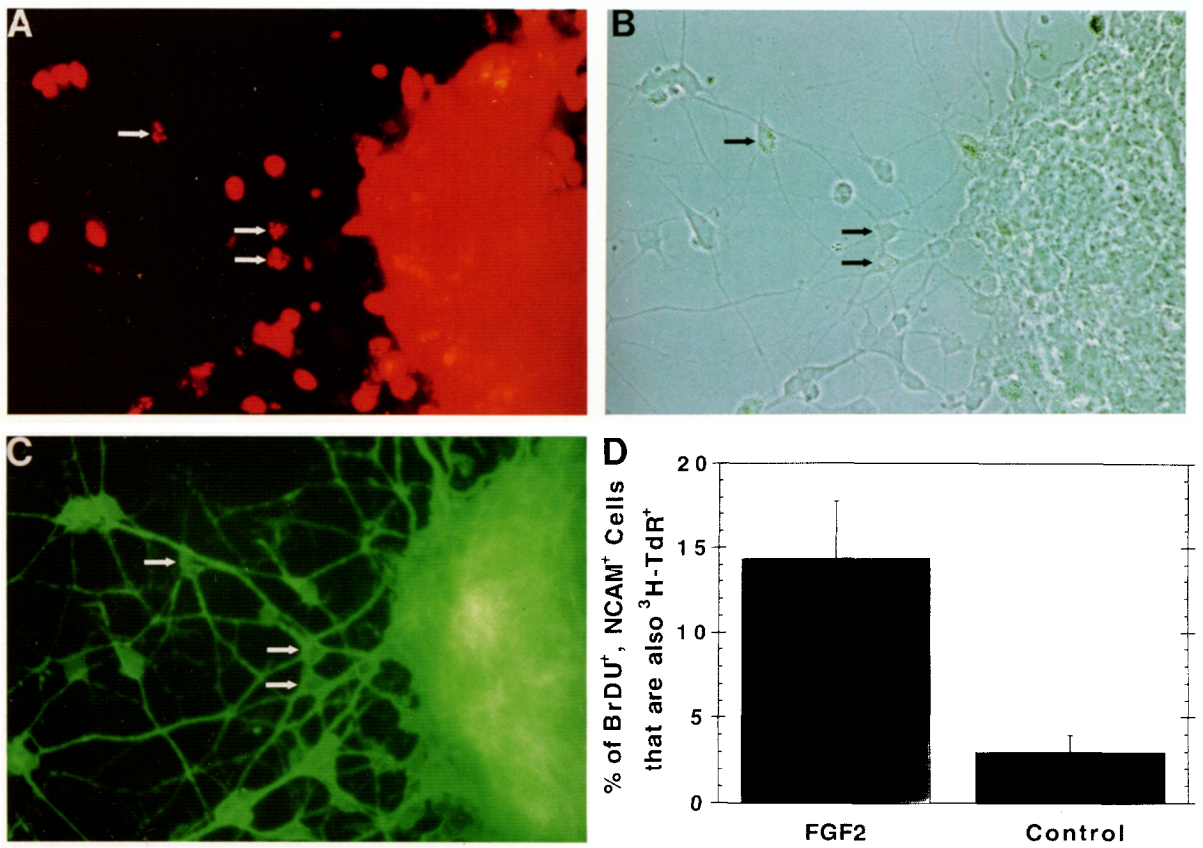

Figure 4. FGF2 Promotes Multiple Divisions of Olfactory Neuron Precursors

OE explant cultures were labeled sequentially with BrdU and ^{3}H-TdR to detect successive cellular divisions.

(A–C) Cultures were grown continuously in FGF2 (10 ng/ml) and incubated with BrdU (1:10,000) for 6 hr, followed by 15 hr in unlabeled TdR (50 μM). A second 6 hr pulse of ^{3}H-TdR (1 μCi/ml) was then administered, followed by 13 hr in unlabeled TdR (50 μM). Cells were then fixed (total time in culture, 41 hr) and processed for autoradiography and BrdU and N-CAM immunocytochemistry. Arrows indicate 3 cells that were labeled with BrdU, ^{3}H-TdR, and N-CAM. The presence of all three markers indicates that these ORNs are the progeny of precursors that went through two rounds of division before undergoing neuronal differentiation. (A) Rhodamine optics showing BrdU immunoreactivity. (B) Phase-contrast optics showing silver grains over cells that incorporated ^{3}H-TdR. (C) Fluorescein optics showing N-CAM immunoreactivity.

(D) Cultures grown in FGF2 (10 ng/ml) or no growth factor were incubated for 12 hr in BrdU (1:10,000), followed by 12 hr in unlabeled TdR (50 μM), then 24 hr in ^{3}H-TdR (0.1 μCi/ml). At t = 48 hr, cultures were fixed and processed for autoradiography and BrdU and N-CAM immunocytochemistry. A minimum of 100 BrdU$^+$, N-CAM$^+$ cells were counted at 6300× magnification in each culture. Bars represent mean ± SEM.

hr (Figure 5A, inset). Assuming these estimates to be correct, then the interval between successive S phases for cycling INPs should be ~9 hr. Thus, the 12 hr gap between BrdU and ^{3}H-TdR pulses in Figure 4D was sufficient to ensure that separate, successive S phases were labeled.

To Exert Its Effects, FGF Must Be Present by a Particular Stage in the Precursor Cell Cycle

The information obtained in Figure 5A was used to determine when in the INP cell cycle FGF must be present to exert its effects. In these experiments, a number of identical OE cultures were pulsed with BrdU and then, 15 hr later, with ^{3}H-TdR, to allow precursor cells undergoing two rounds of division to be detected. FGF2 was applied to different cultures at later and later times, and all cultures were fixed at the end of the ^{3}H-TdR pulse (2 days in culture total).

The results (Figure 5B) demonstrate a 3-fold increase in the number of cells that were the products of two divisions when FGF2 was continuously present versus cultures in which it was never present. The dependence of this effect on time of FGF2 addition suggests that the majority of precursors lose the ability to respond to FGF at a specific time after they exit the first (BrdU-labeled) S phase. Alignment of this curve with the information on the INP cell cycle obtained from Figure 5A suggests that this commitment not to divide again (estimated as the half-maximal point on the curve) occurs slightly more than halfway through the window between the end of the first S phase and the beginning of the second S phase. If we assume

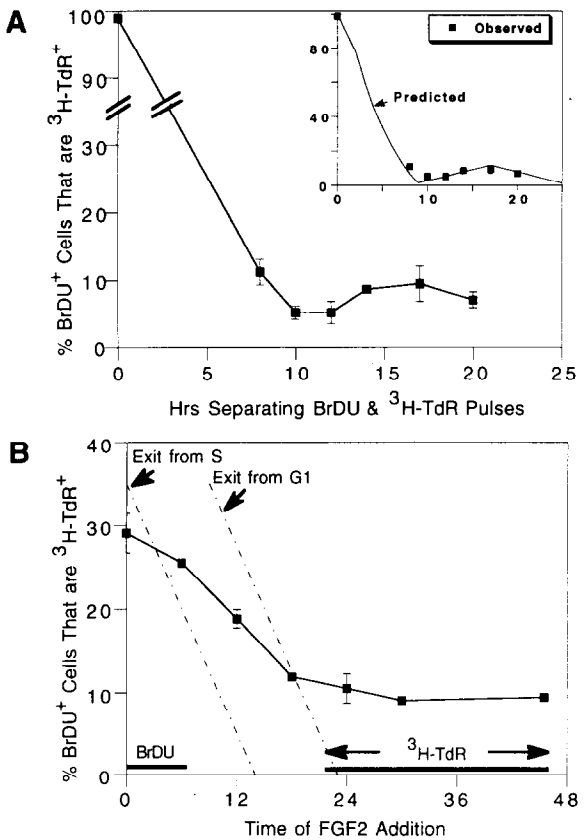

Figure 5. Analysis of FGF2 Effects on Cell Cycle Progression by Olfactory Neuron Precursors

(A and B) A minimum of 200 BrdU$^+$ cells in ten fields were counted in each culture. Data points represent the mean ± range of two cultures.

(A) Explant cultures were grown in FGF2 (10 ng/ml) and pulsed from t = 4 to 6 hr with BrdU (1:5000), then either fixed or chased with unlabeled TdR. At the indicated times, cultures were pulsed for 2 hr with ^{3}H-TdR (5 μCi/ml) and fixed. Times on the abscissa represent the interval between the ends of the BrdU and ^{3}H-TdR pulses.

(Inset) The data from (A) are compared with a predicted curve based on a model of cell cycle phase lengths, the parameters of which were adjusted to provide a close fit to the experimental data (for model derivation, see Experimental Procedures). Predicted values are S = 8 hr, G2 + M = 3.5 hr, total cell cycle length = 17 hr, and 12% of precursors divide twice. The predicted curve is defined by a quotient, the numerator of which is the sum of two components: the first has a value of $(L_{s'} - t)/L_c$ for $t \leq L_{s'}$ and 0 for $t > L_{s'}$; the second has the value 0 for $t \leq L_c - L_{s'}$; $2f(L_{s'} - L_c + t)/L_c$ for $L_c - L_{s'} < t \leq L_c$; $2f(L_{s'} + L_c - t)/L_c$ for $L_c < t < L_c + L_{s'}$; and zero for $t > L_c + L_{s'}$. The denominator of the quotient has a value of L_s/L_c for $t \leq L_{G2M} + Q$; a value of $(L_{s'} + t - L_{G2M} - Q)/L_c$ for $L_{G2M} + Q < t \leq L_{G2M} + Q + L_{s'}$; and a value of $2L_s/L_c$ for $t > L_{G2M} + Q + L_{s'}$. In these equations, t is the time interval between BrdU and ^{3}H-TdR pulses; L_c is the total length of the cell cycle; $L_{s'} = L_s + L_{pulse} - 2Q$, where L_s is the length of S phase, L_{pulse} is the length of the BrdU pulse (2 hr), and Q is the interval over which a cell must be exposed to BrdU or ^{3}H-TdR to become detectably labeled; L_{G2M} is the combined length of G2 and M phases; and f is the fraction of BrdU$^+$ cells that undergo a second cell cycle.

(B) Cultures were pulsed with BrdU from t = 0 to 6 hr, chased with unlabeled TdR for 15 hr, pulsed with ^{3}H-TdR from t = 21 to 45 hr, then fixed and processed. FGF2 (10 ng/ml) was added to a series of separate, duplicate cultures at 6 hr intervals from 0 to 30 hr in culture. Dotted lines illustrate the anticipated progression of BrdU-labeled cells through the cell cycle, from the parameters given above. Because the population of BrdU$^+$ cells ranges from those that were exiting S phase at t = 0 to those just entering S at t = 6 hr, slanted lines are required to illustrate the time span over which the entire population of labeled cells traverses cell cycle phase boundaries.

Table 3. Decline in Average Numbers of Proliferating INPs per Explant in OE Explant Cultures

^{3}H-TdR Labeling Period in Culture	^{3}H-TdR$^+$ Migratory Cells per 30,000 μm^2 Explant	
	No Growth Factor	FGF2
24–48 hr	15.60 ± 1.11 n = 100	50.03 ± 3.01 n = 100
48–72 hr	1.91 ± 0.29 n = 60	8.69 ± 2.30 n = 60
72–96 hr	ND	3.69 ± 1.37 n = 35

Explant cultures were pulsed with ^{3}H-TdR for the indicated 24 hr period, then fixed and processed for autoradiography. Data shown are the mean numbers of ^{3}H-TdR$^+$ cells (±SEM) surrounding each explant, normalized to the average explant area of 30,000 μm^2 (cf. legend to Figure 1). n, number of explants analyzed in each condition.

that the G2/M phase of the cell cycle is relatively short compared with G1, as appears to be the case in most mammalian somatic cells (Baserga, 1985; Murray and Hunt, 1993), this would put the commitment point for FGF responsiveness in early G1.

FGF Effects at Later Times in Culture Suggest a Longer-Lasting Action, as well as the Existence of a Rare Neuronal Progenitor

The results above indicate that FGFs extend neurogenesis in vitro by allowing INPs to undergo one extra round of division before terminally differentiating. This conclusion implies that the actions of FGF in vitro should be transitory; i.e., neurogenesis should be extended, but only by ~17 hr, the length of one INP cell cycle. Table 3 indicates that, on average, this is largely true. When OE explant cultures are labeled with ^{3}H-TdR, not from 24 to 48 hr (as in Figure 1), but rather from 48 to 72 or 72 to 96 hr, there is a rapid decline in proliferation among the migratory cells to very low levels, even in the presence of FGF2.

Interestingly, however, in FGF-treated cultures, occasional explants can be found—even when labeling is carried out from 72 to 96 hr—that contain large numbers of ^{3}H-TdR$^+$ cells. This observation raises the possibility that, even though proliferation declines steadily in OE cultures on the whole, it may continue unabated in a small fraction of explants, at least when FGFs are present. To test this idea, the raw data that were averaged to produce Table 3 were plotted instead as frequency histograms, to reveal the percentages of explants with labeling indices falling within different ranges (Figure 6).

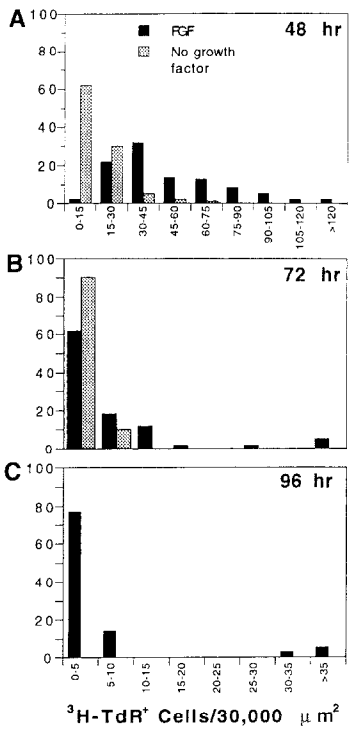

Figure 6. Effects of FGF2 on Distribution of Explant Labeling Indices at Late Times in Culture

OE explants were cultured in the presence or absence of FGF2 (10 ng/ml) for either 48, 72, or 96 hr and were exposed to ^{3}H-TdR (0.1 μCi/ml) during the last 24 hr 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 explant area. As in Table 1 and Table 3, the unit of area used in normalization was 30,000 μm^2, the average explant size. Ordinate scale is percentages of explants exhibiting particular ranges of explant labeling indices.

(A) Cultures (48 hr) in the presence versus absence of FGF2; 100 explants were analyzed in each condition.

(B) Cultures (72 hr) in the presence versus absence of FGF2; 60 explants were analyzed in each condition. Note that the bin sizes are smaller than those in (A).

(C) Culture (96 hr) grown in the presence of FGF2; 35 explants were analyzed. Bin sizes, same as in those in (B).

It was noted that if, on average, explant labeling indices were not independent of explant area, then normalization to explant area would introduce a systematic bias. Linear regression analysis of the unbinned data revealed a small but significant ($p < .05$) negative correlation between (normalized) explant labeling index and explant area (i.e., smaller explants tended to have larger normalized labeling indices). The bias introduced by this correlation was small, however, and when the unbinned data were corrected to remove it, rebinned, and plotted as in (A)–(C), the distributions were not substantially altered (data not shown).

The results indicate that, in the absence of added growth factor, labeled cells are distributed among explants in a unimodal fashion that over time merely shifts to the left (i.e., fewer labeled cells per explant are seen; note the change in bin sizes from 48 hr [Figure 6A] to 72 and 96 hr [B and C]). In contrast, in the presence of FGF2, the distribution of labeled cells among explants varies much more widely even at 48 hr, and by 72 hr, it is distinctly bimodal, with 93% of explants containing ≤20 labeled cells per 30,000 μm^2 (mean = 4.79 cells), but a small fraction (~7%) contain >25 labeled cells (mean = 63.35 cells). This distinction is even sharper at 96 hr in the presence of FGF2, with 91% of explants containing ≤10 labeled cells per 30,000 μm^2 (mean = 1.50 cells), while ~8.5% contain >30 labeled cells (mean = 50.25 cells).

In FGF2 then, a small fraction of explants continue to produce large numbers of cells that can take up ^{3}H-TdR as late as 72–96 hr in vitro. The fraction of explants that exhibit this capacity appears to be maintained at the level of ~7%–8% of explants. Importantly, a substantial number of ^{3}H-TdR-incorporating cells in this population of explants continue to give rise to ORNs. This was determined by a pulse-chase experiment in which OE explant cultures, grown continuously in FGF2, were labeled from 48 to 70 hr with ^{3}H-TdR, chased for 12 hr with unlabeled TdR, and then processed for N-CAM immunocytochemistry and autoradiography.

Of 100 explants analyzed, 5 had labeling indices of >25 cells per 30,000 μm^2 (mean = 113.2 cells; 1 of these explants is shown in Figure 7). For each of these 5 explants, all migratory, ^{3}H-TdR$^+$ cells were scored for N-CAM immunoreactivity. The percentage of ^{3}H-TdR$^+$ cells that were N-CAM$^+$ varied from 53% to 100% (mean = 78, SD = 18.1). These data indicate that, among the small fraction of explants that still exhibit proliferation at late times in FGF2-treated cultures, a large proportion of the proliferating, migratory cells give rise to ORNs.

Overall, the data in Figure 6 and Figure 7 are consistent with the view that a small fraction of explants (5%–8%) undergo continuous ORN production for up to 4 days in vitro (the latest time examined so far), in the presence—but not in the absence—of FGF2. Why these explants are different from the rest (for which neuron precursor proliferation essentially ceases, even in FGFs) is not clear, but one possible interpretation is that cells that can be stimulated by FGF2 to undergo continued proliferation in vitro may also be rare in the OE in vivo, such that they are not present in most explants. The apparent rarity of such cells, coupled with the observation that they do not appear to behave like typical INPs (which undergo one or two divisions and then produce postmitotic neuronal progeny) suggests that they may lie significantly upstream of INPs in the neuronal lineage. This point, and its implications, are discussed below.

Discussion

FGFs Prolong Neurogenesis by the OE In Vitro

The fact that the OE, a tissue that undergoes continual neurogenesis in vivo, rapidly ceases to do so in vitro in defined serum-free medium has provided a means to screen for growth factors that play a role in control of neurogenesis. Using an assay designed to detect increases in "late" proliferation of neuronal precur-

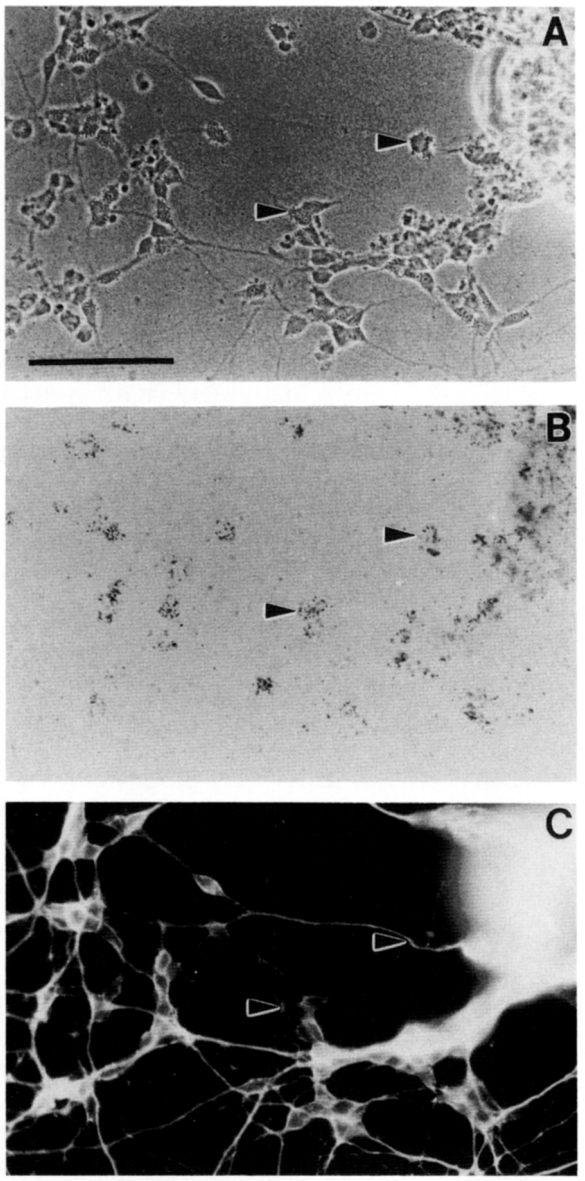

Figure 7. Explants That Exhibit Late Proliferation in Response to FGF2 Give Rise to Neurons

Cultures were grown continuously in FGF2 and pulsed with ^{3}H-TdR (0.1 μCi/ml) from 48 to 70 hr, then chased with unlabeled TdR (50 μM) for 12 hr and fixed and processed for N-CAM immunocytochemistry and autoradiography. ^{3}H-TdR labeling indices were calculated (as in Figure 6) for 100 explants in 5 cultures. The distribution of labeling indices in this experiment was similar to that observed at t = 72 hr with no chase (Figure 6B): 95% of explants had labeling indices <20 (mean = 4.22), while 5% had labeling indices >25 (mean = 108.1). Photographs show a portion of 1 of these explants and cells that have migrated from it; this explant's ^{3}H-TdR labeling index was 329 labeled cells per 30,000 μm^2 (94 ^{3}H-TdR$^+$ cells surrounding an explant with 8565 μm^2 area). (A) Phase-contrast optics. (B) Bright field optics showing silver grains over the nuclei of cells that incorporated ^{3}H-TdR. (C) Rhodamine optics showing N-CAM immunoreactivity. Note the large numbers of ^{3}H-TdR-labeled cells that are also N-CAM$^+$ by the end of the chase, as well as the occasional ^{3}H-TdR$^+$ progenitor cells that are N-CAM$^-$ (arrowheads). Bar, 50 μm.

sors, several members of the FGF family were found to prolong neurogenesis signficantly in vitro. FGF2, the FGF family member that was studied in detail, had two actions: the first was to increase the number of INPs that underwent two, rather than one, rounds of cell division prior to giving rise to ORNs; the second action was to maintain, in a distinct, small fraction of explants, the continued proliferation or survival of neuronal precursors for as late as 72–96 hr in culture (the longest time tested).

The conclusion that FGF2 causes some INPs to undergo an additional cell division in vitro is supported by the following observations: FGF2 causes a delay in the onset of N-CAM expression by the progeny of INPs (Figure 3), FGF2 does not alter the ^{3}H-TdR pulse-labeling index of INPs at early times in culture (Table 1), and FGF2 increases by 3- to 5-fold the fraction of ORNs that are double-labeled by short pulses of BrdU and ^{3}H-TdR administered 12 hr apart (Figure 4; Figure 5B), an interval sufficiently long so that double labeling implies traversal of two successive S phases in vitro (Figure 5A). In addition, it was noted that, in the presence of FGF2, the migratory cells labeled by ^{3}H-TdR administered between 24 and 48 hr were sometimes found in small clusters of 4 or more cells (Figure 1B); such clusters would be expected if some INPs had divided twice and their clonal descendants had not migrated far from each other.

The conclusion that FGFs allow neurogenesis to be maintained in a small fraction of OE explants is based on the analysis of the number of migrating cells per explant that could be labeled by ^{3}H-TdR at increasingly late times in culture (Figure 6). It was observed that, in the presence of FGF2, 5%–8% of explants continued to produce large numbers of dividing, migratory cells long after proliferation had virtually ceased in the remaining explants. In addition, a large proportion of the proliferating migratory cells produced by such explants gave rise to N-CAM$^+$ neurons (Figure 7).

What Is the Mechanism of Action of FGFs?

The early effects of FGFs (i.e., those observed between 24 and 48 hr) are most likely a direct action on INPs, since they are seen in dissociated cultures that are free of basal cells (Table 1E). The fact that these effects can be accounted for by an increase in the fraction of INPs that undergo a second cell division in vitro (Figure 4; Figure 5), rather than exiting the cell cycle and expressing N-CAM (Figure 3), suggests that the mechanism of action of FGFs may be to repress terminal (neuronal) differentiation, thereby making further divisions possible. The data in Figure 5 also suggest that this action of FGFs must be exerted on INPs by early G1 of their cell cycle, a time at which commitment to terminal differentiation would be expected to occur (Soprano and Cosenza, 1992). This mechanism of action of FGFs on INPs bears similarities to the way FGFs are believed to act on muscle cell precursors. In mouse MM14 myoblasts, for example, FGFs promote myoblast proliferation not by acting as mito-

gens but by repressing terminal differentiation in G1, thereby allowing progression of cells through additional cell cycles (Clegg et al., 1987). It may be the case that FGFs act through similar mechanisms in the immediate precursors of many kinds of differentiated, postmitotic cells.

Although the above discussion casts the role of FGFs in terms of repressing differentiation of INPs into postmitotic ORNs, the data cannot rule out the possiblity that, for INPs, the "terminal" differentiation step being repressed by FGFs is actually apoptosis (programmed cell death). In theory, some or all of the INPs that undergo a second round of division in the presence of FGFs could actually be cells that were fated to die, rather than to give rise to neurons. However, this seems unlikely. First, we found that FGF2 treatment increased the ratio of the number of INPs that divide twice to the total number of INPs (Figure 4; Figure 5B). If FGFs acted by preventing cell death, then to be consistent with these data, FGF2 would have had to promote selectively the survival of only those INPs that could undergo a second round of division, or no change in this ratio would have been observed. Second, we have recently combined the use of an in situ labeling technique that detects apoptotic cells (Gavrieli et al., 1992) with pulse-chase ^{3}H-TdR incorporation analysis, to determine directly whether apoptosis is the fate of INPs in vitro. We find that the fraction of INPs that undergo apoptosis is very small: ~0.12% of INPs are apoptotic at the time of ^{3}H-TdR incorporation, and only ~6% of their progeny are apoptotic 20 hr later. Moreover, the number of INPs that undergo apoptosis is not changed significantly by the presence of FGF (data not shown). Thus, we believe that the direct actions of FGFs on INPs are not the result of effects on cell survival.

The INPs of ORNs Exhibit Characteristics of Transit Amplifying Cells

The fact that FGF2 drives some INPs to undergo an additional cell division in vitro, but does not divert the progeny of those cells from a neuronal fate, suggests that INPs may be committed progenitors with an inherently limited capacity for self-renewal. Interestingly, in vivo studies by MacKay-Sim and Kittel (1991) provide additional, if indirect, support for this idea: their analysis of ^{3}H-TdR incorporation and dilution by ORN precursors in adult mouse OE indicated that the immediate progenitors of ORNs are rapidly dividing cells that divide only two, or occasionally three, times before undergoing terminal differentiation.

Several characteristics of INPs—apparent restriction of developmental fate and limited proliferative capacity as well as regulation of proliferation by growth factors—typify transit amplifying cells. In other self-renewing tissues, such as epidermis and the hematopoietic system, transit amplifying cells occupy a position between undifferentiated stem cells and the mature, functional cells that characterize the tissue (Hall and Watt, 1989; Potten and Loeffler, 1990).

If INPs function as neuronal transit amplifying cells, then it may be most appropriate to view FGFs as factors which increase the likelihood that these cells complete the fixed number of divisions of which they are intrinsically capable. A consequence of this view is the expectation that not all INPs should exhibit a proliferative response to FGFs, since some of the cells in any collection of INPs should already be only one cell division away from their proliferative limit, and therefore incapable of executing an additional cell cycle. Indeed, since amplifying divisions expand cell numbers exponentially, at any given time transit amplifying cells nearer their proliferative limit should outnumber those that are far from that limit. This may explain why, in the experiments in this study, the fraction of INPs driven to divide twice by FGF2 was sometimes relatively low (e.g., 12% in Figure 5A). It is also possible that other signals, in addition to FGF2, may be needed to ensure that all INPs undergo every division of which they are capable.

Late Effects of FGFs Suggest That Progenitors of INPs Are Present In Vitro

If INPs are transit amplifying cells, then there must be another cell type that acts as a progenitor to INPs. This may be a stem cell (i.e., a self-renewing cell that, on average, generates one INP with each division), or there may be one or more types of transit amplifying cells interposed between the INP and its stem cell. At present, there is no way to know how many divisions lie between ORNs and their ultimate progenitors. However, the more divisions there are, the less abundant the earliest progenitors need be, to be able to generate the full complement of ORNs. Consequently, it is possible that some cells sufficiently far upstream of INPs in the ORN lineage are so rare that they might occur in only a fraction of OE explants. If such cells require FGFs in order to proliferate (or to survive), then that circumstance could provide a simple explanation for the observation that only 5%–8% of OE explants appear to be capable of long-term, FGF-driven neurogenesis (Figure 6; Figure 7). At present, however, we cannot rule out the alternative possibility that these explants do not contain a rare type of stem cell or neuronal progenitor, but instead differ from the majority of explants in another way, e.g., by creating a local environment that acts in combination with FGFs to extend the proliferative capacity of INPs or INP progenitors.

Whether the cell type affected by FGF2 in these explants is a stem cell or an amplifying cell may not become clear until much longer-term cultures are studied. However, the data suggest the possibility that neurogenesis in the OE may be a multistep process involving several distinct progenitor cell types, a view of neurogenesis that parallels observations in other stem cell systems (Hall and Watt, 1989; Potten and

Loeffler, 1990). It will be interesting to determine whether markers that distinguish among neuronal precursors at different stages in neuronal lineages can be obtained. We have found that one molecule known to be important for ORN production in vivo, the transcription factor MASH1 (Guillemot et al., 1993), is expressed in only a subset of the migratory, N-CAM$^-$ cells associated with OE explants in vitro, and that these cells proliferate (Calof et al., 1994b; data not shown). It is not yet clear, however, whether expression of this polypeptide marks a distinct position in the ORN lineage.

In Vivo Significance of FGF Effects on Neurogenesis

Information on expression patterns of FGFs in the developing embryo is incomplete, with only some of the 9 known FGFs having been examined so far (reviewed by Baird, 1994). Nonetheless, it is already clear that several FGFs are present in locations where they could potentially influence OE neurogenesis in vivo. For example, FGF2 immunoreactivity has been seen in mesenchyme underlying the OE (as well as in numerous basement membranes) in the rat embryo at E18, a comparable age to the E16 mouse (Gonzalez et al., 1990). FGF7 is expressed in the stroma underlying OE in mouse beginning at E14.5 (Mason et al., 1994), and FGF1 immunoreactivity has been shown around OE in E19 rat (Fu et al., 1991). A characteristic of the FGF family, also known as the heparin-binding growth factor family, is that its members are thought not to be freely diffusible in vivo but rather to be bound to, and presented to cells by, heparin sulfate proteoglycans in extracellular matrix and on cell surfaces (Baird, 1994). It is tempting to speculate that the presence of FGFs in OE basement membrane contributes to the preferential localization of neuronal precursor proliferation in the basal region of adult OE.

The fact that FGFs can potentially be presented to cells when adsorbed to cell surfaces (as can other heparin-binding growth factors [e.g., Ratner et al., 1988]) also raises the interesting possibility that the presence of endogenous FGFs may contribute to a dependence of neuronal precursor proliferation on cell-cell contact. Such a dependence has been seen in in vitro systems other than OE (e.g., Watanabe and Raff, 1990; Gao et al., 1991; Temple and Davis, 1994), including one (embryonic rat retina) in which the stimulatory effects of high cell density could be mimicked by FGF1 or FGF2 (Lillien and Cepko, 1992). That cell contact stimulates neurogenesis in cultured OE is almost certainly the case as well, as is apparent from the data in Table 1 (in which the true labeling index of the neuronal cell fraction was 8-fold lower for purified, dissociated neuronal cells [column E] than for those in explant cultures [column A]). Moreover, the fact that FGF2 restored the labeling index of dissociated neuronal cells to approximately that seen in explant cultures grown in the absence of growth factors (Table 1) is consistent with the possibility that endogenous FGFs may mediate some of the effects of cell contact and/or density on OE neurogenesis.

Effects of Other Factors on Neurogenesis in the OE

Two other groups have reported stimulatory effects of factors other than FGFs on neuronal production by cultured OE. Pixley (1992) reported that coculture with CNS astrocytes could prolong neurogenesis by neonatal rat OE. Since astrocytes are known to produce FGFs (Woodward et al., 1992; Baird, 1994), it is possible that FGFs account for some or all of the effects observed by this investigator. Recently, Mahanthappa and Schwarting (1993) reported that TGFβ2 stimulates neurogenesis in postnatal rat OE cultures, an effect that was not observed in the present study (Figure 1E). There are several possible explanations for this difference. First, these investigators did not rule out the possibility that TGFβ2 was actually acting as a survival factor specifically for newly generated neurons (only the lack of an effect of TGFβ2 on the total number of cells per culture was reported). Second, growth factor responsiveness of the OE may change between mid-gestation (the time at which OE tissue was taken in the present study) and postnatal day 3–4 (the age used by Mahanthappa and Schwarting). Finally, it may be the case that, in the present study, precursor proliferation was unaffected by TGFβ2, not because cells were unable to respond to this molecule but because they were already being maximally stimulated by it. It is known that cells of embryonic OE contain TGFβ2 mRNA in vivo (Millan et al., 1991), and it can be concluded from data presented by Mahanthappa and Schwarting that postnatal rat OE cultures produce TGFβ2 in sufficient quantity to support a substantial level of neuronal production (although not as much as when exogenous TGFβ2 was added). For a variety of reasons—different tissue age, different species, or different culture conditions—it is possible that the cultures used in the present study might contain a higher concentration of endogenous TGFβ2 than those used by Mahanthappa and Schwarting. It will be interesting to test this latter possibility directly.

Effects of Growth Factors on Neurogenesis in Other In Vitro Systems

Several groups have reported that FGFs stimulate in vitro proliferation of progenitor cells from several regions of embryonic nervous system, including E10 telencephalon (Murphy et al., 1990; Kilpatrick and Bartlett, 1993), cerebral cortex (Gensburger et al., 1987), corpus striatum (Cattaneo and McKay, 1990; Vescovi et al., 1993), hippocampus (Ray et al., 1993), and retina (Lillien and Cepko, 1992). These findings, together with the observation of FGFR1 expression in germinal zones throughout embryonic brain (Heuer et al., 1990), suggest that FGF effects on neurogenesis may be widespread. Growth factors other than FGFs also influence proliferation and differentiation of neural precursors in vitro: EGF and TGFα stimulate prolifera-

tion of embryonic retinal and striatal progenitor cells, which give rise to neurons and glia (Anchan et al., 1991; Lillien and Cepko, 1992; Reynolds et al., 1992). Both EGF and insulin can stimulate proliferation of rat sympathetic ganglion neuron precursors (DiCicco-Bloom et al., 1990). NGF can act in concert with other factors to promote proliferation of progenitor cells from embryonic striatum (Cattaneo and McKay, 1990), and neurotrophin 3 (NT-3) promotes proliferation of cultured neural crest cells (Kalcheim et al., 1992). In contrast, CNTF apparently inhibits the proliferation of sympathetic neuron progenitors (Ernsberger et al., 1989). In many of these systems, it remains to be sorted out whether the growth factors that affect neuronal production do so by acting as mitogens for neuronal precursors, inhibiting terminal differentiation of neuronal precursors, promoting survival of neuronal precursors (Birren et al., 1993; DiCicco-Bloom et al., 1993), influencing fates chosen by bi- or multipotential neural precursors (Sieber-Blum, 1991; Anderson, 1993; Shah et al., 1994), or even promoting survival of newly generated neurons.

In this regard, the simplicity of the OE as an experimental system has proved to be a significant advantage. The fact that proliferating neural precursors in the OE seem to be committed to giving rise to a single type of differerentiated cell, the ORN, has made it possible to draw conclusions about the times and mechanisms of action of FGFs within this neuronal lineage. Interestingly, the conclusions that we have reached—that INPs behave as transit amplifying cells and that FGFs act both on INPs and another cell that may lie far upstream of INPs—suggest that the progression from stem cell to ORN may involve several distinct cellular stages. Although evidence supporting the notion of multiple steps in neural lineages has been obtained in other in vivo and in vitro systems (e.g., Takahashi et al., 1994; Anderson, 1993), these steps have been thought to parallel the progressive restriction of a multipotential precursor's fate (cf. Anderson, 1993). In the OE, where no evidence for multipotentiality of neuronal precursors has yet been obtained, the existence of such steps may have more to do with intricacies in the regulation of cell number, rather than cell fate. This idea recalls aspects of the hematopoietic system, in which some unipotential lineages (e.g., the erythroid lineage) progress through multiple precursor stages, each of which exhibits different growth factor requirements (Dexter and Spooncer, 1987).

Experimental Procedures

Materials

Recombinant human NGF, brain-derived neurotrophic factor (BDNF), NT-3, and recombinant rat CNTF were obtained from Genentech (generous gifts of David Shelton, John Winslow, Karoly Nikolics, and Gene Burton). Recombinant human acidic FGF (FGF1), basic FGF (FGF2), TGFα, EGF, and platelet-derived growth factors AA and BB (PDGF-AA and PDGF-BB) were from US Biochemicals. Recombinant human TGFβ1 and TGFβ2 were from Genzyme. Recombinant human keratinocyte growth factor (KGF or FGF7) was from Collaborative Research. Recombinant human FGF4 was from R & D Systems. Growth factors were stored at −85°C in concentrated stocks made up in 1 mg/ml Clinical Reagent Grade bovine serum albumin (CRG-BSA; ICN Biochemicals) in calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS). BrdU was from Amersham (cell proliferation labeling reagent #RPN201), and ^{3}H-TdR (70–90 Ci/mmol) was from New England Nuclear. NTB2 emulsion, D-19 developer, and fixer were from Kodak. Tissue culture media, antibiotics, and merosin (human) were from GIBCO-BRL. Unless otherwise noted, all other reagents were from Sigma.

Tissue Culture

OE was purified as described (Calof and Chikaraishi, 1989) from embryos of CD-1 mice (Charles River) at E14.5–E15.5, for which vaginal plug date was designated as E0.5. For explant cultures, pieces of purified OE were plated onto acid-washed glass coverslips (12 mm, #1 thickness; Propper) that had been coated with poly-D-lysine (1 mg/ml in water) followed by merosin (10 μg/ml in CMF-HBSS). Washed coverslips were transferred to 24 well tissue culture trays (Falcon) and covered with defined, serum-free low calcium culture medium (LCM) prepared as described (Calof and Lander, 1991), except that BSA was reduced to 1 mg/ml CRG-BSA. For some experiments, a dissociated neuronal cell fraction (INPs + ORNs) was prepared from 6 hr OE suspension cultures as described (Calof and Lander, 1991; Calof et al., 1994a). These cells were plated in LCM at ~2 × 10^5 cells per well in poly-D-lysine-coated 96 well tissue culture trays (Costar).

Immunocytochemistry and Autoradiography

For visualizing N-CAM, cultures were fixed in room temperature acetone (5 min) or Omnifix II (10 min; AnCon Genetics) and stained with monoclonal antibody AG1 (DiFiglia et al., 1989), using either ascites fluid (1:500) followed by rhodamine goat anti-mouse IgG (1:100; Tago) or biotinylated AG1 IgG (10 μg/ml) followed by fluorescein isothiocyanate Z-avidin (1:50; Zymed). Alternatively, monoclonal rat anti-N-CAM H28 (kind gift of Christo Goridis, INSERM-CNRS, Marseilles, France; Gennarini et al., 1984) was used as full-strength hybridoma supernatant applied to cells fixed for 10 min in 3.7% formaldehyde/5% sucrose in Dulbecco's phosphate-buffered saline (PBS) followed by Texas red goat anti-rat IgG at 1:50 (Jackson). For staining with monoclonal rat anti-BrdU (Sera-Lab clone BU1/75 [ICR 1]; Accurate), cultures were fixed in Omnifix II, permeablized in 0.1% Triton X-100 in PBS, treated with 2 N HCl in water for 15 min, and incubated overnight in anti-BrdU ascites (1:500). BrdU staining was visualized with Texas red goat anti-rat IgG (Jackson; 1:50) or, in cultures grown on plastic dishes, with biotinylated rabbit anti-rat IgG (2.5 μg/ml; Vector) followed by avidin-horseradish peroxidase (Vectastain ABC-peroxidase kit). Cultures incubated in ^{3}H-TdR were either fixed and processed for immunocytochemistry as specified above or simply fixed in 3.7% formaldehyde/5% sucrose in PBS. Coverslips were dehydrated and dipped in NTB2 emulsion diluted 1:1 in water, then exposed at −85°C. Cultures pulsed for 2 or 6 hr were exposed for 2 days; those pulsed for 24 hr pulse were exposed for 7 or 8 days. Slides were then developed in D-19 developer, and nuclei were stained with Hoechst 33258 (bisbenzimide; 1 μg/ml).

Analysis of FGF Receptor Expression

Total RNA (9–10 μg) was isolated from purified E14.5–15.5 mouse OE or from the dissociated INPs + ORNs cell fraction using the method of Chomczinski and Sacchi (1987). RNA was added to RT buffer (GIBCO-BRL) containing 1 mM dNTPs, 200 pmol of random hexamer primer (Pharmacia), and 10 mM dithiothreitol (total volume, 38 μl). To half this mixture, 200 U (1 μl) of MMuLV RT (GIBCO-BRL) was added, then incubated at 37°C for 1 hr. The remaining half was saved as a no-RT control. Then, 2 μl of each mixture was added to 18 μl of buffer (50 mM KCl, 10 mM Tris–Cl [pH 8.3], 1.5 mM $MgCl_2$, 0.001% gelatin) containing 250 μM dNTPs, 0.25 μM each of forward and reverse primers, and 0.125 U/μl of Taq DNA polymerase (Perkin-Elmer). Cycling parameters were denaturation at 95°C for 45 s, annealing at 55°C for

30 s, and elongation at 72°C for 1 min. Primers corresponded to transmembrane regions of the receptors (Lee et al., 1989; Dell and Williams, 1992; Ornitz and Leder, 1992; Stark et al, 1991): FR1, 5′-AGAGACCAGCTGTGATGA-3′ (forward) and 5′-GGCCACTTTGGTCACACG-3′ (reverse); FR2, 5′-GAGAGAAGGAGATCACGG-3′ (forward) and 5′-TGCCACGGTGACCGCCTC-3′ (reverse); FR3, 5′-TGATGGAAACTGATGAGG-3′ (forward) and 5′-GGCCACGGTGACAGGCTT-3′ (reverse); FR4, 5′-CGTGGACAACAGCAACCC-3′ (forward) and 5′-AGCCACGGTGCTGGTTTG-3′ (reverse). Mixtures (15 μl of each) were analyzed on 1.3% agarose gels and visualized with ethidium bromide. Amplification products were subcloned into a SmaI site in pSport (GIBCO-BRL) and sequenced to confirm identities (Sequenase 2.0; US Biochemicals).

Estimations of Cell Cycle Parameters

The predicted curve in Figure 5 (inset) was obtained by fitting data to a simple model, in which the fraction of $BrdU^+$ cells that are $^3H\text{-}TdR^+$ was calculated for each time point by dividing a predicted number of double-labeled cells by a predicted number of $BrdU^+$ cells. For any population starting with an arbitrary number of cells in the cell cycle (and having the same cell cycle kinetics), the denominator is determined by the length of the cell cycle, the length of the BrdU pulse, the duration of BrdU incorporation required for detectable labeling, and the length of the G2 + M phases of the cell cycle (Nowakowski et al., 1989). The numerator is the sum of two components: cells that are double-labeled because they were exposed to ^3H-TdR before having left the S phase during which they incorporated BrdU and cells that are double-labeled because they were undergoing a second S phase at the time of the ^3H-TdR pulse. Calculating these components is straightforward and requires only introducing a parameter representing the fraction of $BrdU^+$ cells that enter a second cell cycle (rather than becoming postmitotic). In addition, it was assumed that the duration of ^3H-TdR incorporation required for detectable labeling was similar to that required for detectable labeling by BrdU (neither parameter had a substantial impact on the output of the model).

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