Introduction

Sonic hedgehog (SHH) is required during development of the spinal cord for specification of ventral neurons (Briscoe et al., 2001; Wijgerde et al., 2002) and oligodendrocyte progenitors

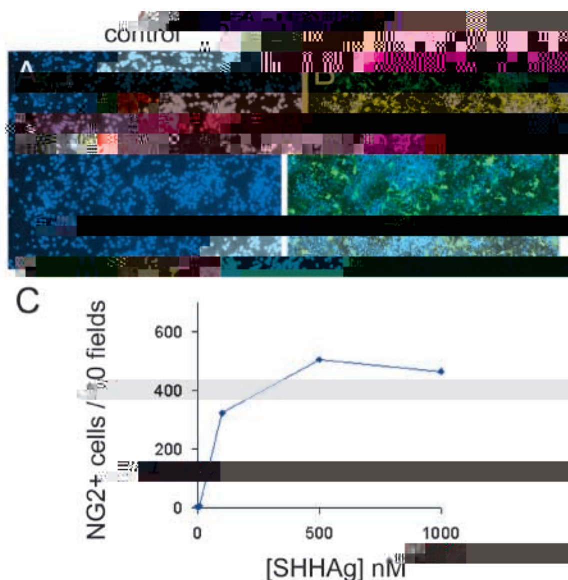

Fig. 2. Induction of OLPs by the Hedgehog agonist SHHAg1.2.Mouse E13.5 neocortical neuroepithelial cells were cultured in the presence or absence of SHHAg for 4 DIV. The cultures were fixed and immunolabelled with polyclonal anti-NG2. (A) Control cultures lacked NG2 immunoreactivity. (B) Numerous NG2-positive cells developed in the presence of 100 nM SHHAg1.2. (C) The dose-response curve shows induction of NG2-positive cells at a half-maximal concentration of SHHAg1.2 of ~25 nM.

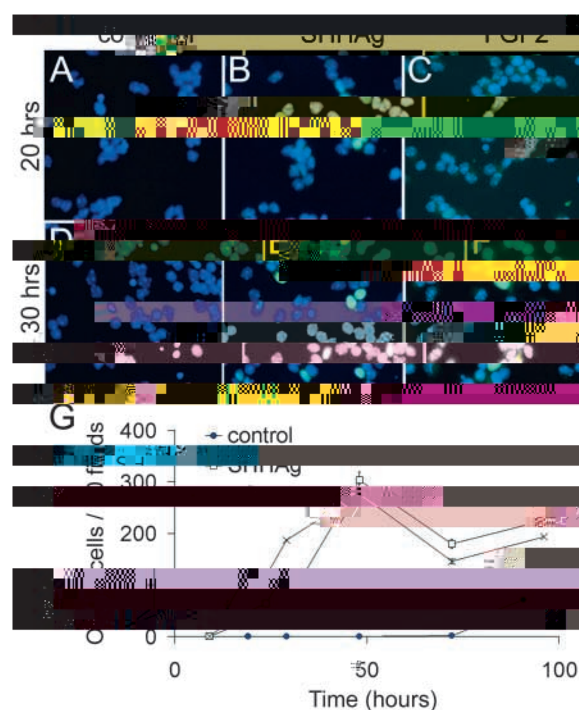

Fig. 3. Rapid induction of OLIG2 by SHHAg or FGF2. Neocortical precursors from E13.5 mice were cultured in the presence or absence of FGF2 or SHHAg and assayed for OLIG2 immunoreactivity at different times. OLIG2-positive nuclei appeared within the first 20 hours in both FGF2-treated (C,F) and SHHAg-treated (B,E)

(Fig. 1F). FGF2 also induced ISL1/2-positive neurons, presumably motor neurons, in chick dorsal spinal cord explants (not shown). (Note that this activity of FGF2 was not inhibited significantly by the Hedgehog inhibitor cyclopamine (Fig. 1F). This is discussed in more detail below, in the section entitled ‘FGF2 dependent induction of OLPs...’.)

In parallel experiments we showed that the SHH agonist Cur-0188168 (ShhAg1.2, hereafter referred to simply as SHHAg) (Frank-Kamenetsky et al., 2002) can induce neocortical precursors to generate OLPs in a dose-dependent manner (Fig. 2). This confirms previous studies with full-length recombinant SHH (Tekki-Kessaris et al., 2001; Alberta et al., 2001; Murray et al., 2002).

As we showed previously (Tekki-Kessaris et al., 2001), cortical cultures maintained in defined medium eventually generate NG2-positive OLPs if left long enough (DIV5-6) even without added growth factors. However, most of this endogenous activity can be neutralised by cyclopamine, demonstrating that it derives mainly from Hedgehog proteins made by the cultured cells (Tekki-Kessaris et al., 2001). In the experiments reported here we used concentrations of FGF2 (10 ng/ml, ~0.6 nM) or SHHAg (100 nM) that induced NG2-positive OLPs by DIV3-4, well ahead of endogenous Hedgehog activity.

Rapid induction of OLIG2 by FGF2 or SHH

Activation of NG2 expression is a relatively late event in oligodendrocyte lineage progression, an earlier lineage marker being OLIG2. We looked at induction of OLIG2 expression in response to FGF2 or SHHAg. OLIG2-positive cells first appeared within 20 hours of either FGF2 or SHHAg treatment, peaking around 48 hours (Fig. 3). Control cultures without added FGF2 or SHH did not develop any OLIG2-positive cells for at least 70 hours (Fig. 3G).

FGF2-mediated induction of OLPs is SHH independent: SHH requires FGFR

The fact that either SHH or FGF2 can induce OLPs raises the question: do these different factors act sequentially in the same induction pathway or in separate, parallel pathways? As an example of sequential action, FGF2 might stimulate cells in the cortical cultures to synthesise SHH or a related Hedgehog protein, which could secondarily induce OLPs. If so, one would expect to be able to block FGF2 activity with cyclopamine, an inhibitor of Hedgehog signalling (Cooper et al., 1998; Incardona et al., 1998). Alternatively, SHH might stimulate synthesis or release of FGF. In that case one would expect to block SHH-mediated induction by PD173074, which inhibits signalling through FGFR (Dimitroff et al., 1999; Skaper et al., 2000). If, on the other hand, SHH and FGF2 trigger independent, parallel pathways, one would not expect to block the SHH effect with PD17074, or the FGF2 effect with cyclopamine.

To test these predictions we cultured E13.5 neocortical precursors at high density in the presence of FGF2 or SHHAg, with or without cyclopamine or PD173074, and looked for induction of OLIG2 at DIV2. We found that the OLIG2-inducing activity of FGF2 (10 ng/ml) was strongly inhibited by PD173074, as expected, but was unaffected by cyclopamine (Fig. 4A,B). Moreover, cyclopamine did not inhibit the OLP-

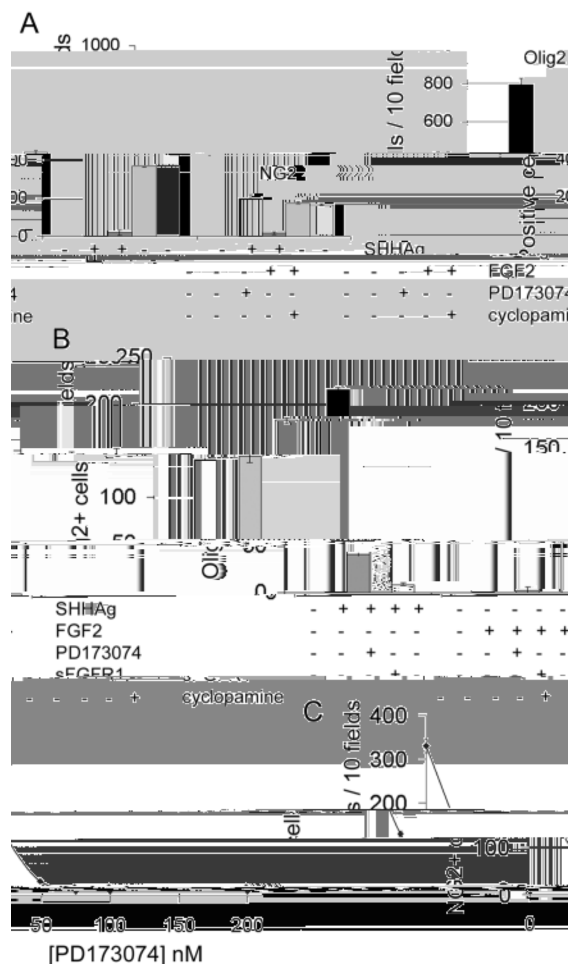

Fig. 4. OLP induction by SHH requires FGFR. (A) E13.5 mouse neocortical cells were cultured for 2 DIV in the presence or absence of FGF2 or SHHAg, with or without the FGFR inhibitor PD173074 or the Hedgehog inhibitor cyclopamine. The cultures were assayed for OLIG2 immunoreactivity at DIV2 or NG2 immunoreactivity at DIV4. Induction of both OLIG2-positive and NG2-positive cells by SHH was inhibited by PD173074. The inducing activity of FGF2 was unaffected by cyclopamine. (B) Cortical cells from mouse E13.5 embryos were cultured in the presence or absence of SHHAg or FGF2, PD173074, a combination of FGFR1αIIIc and FGFR1βIIIc extracellular domains (sFGFR1) or cyclopamine. The inducing effect of FGF2 was inhibited by both PD173074 and sFGFR1 but not by cyclopamine. The effect of SHH was inhibited by PD173074 but not by sFGFR1, suggesting that SHHAg activity requires ligand-independent activation of FGFR. (C) Dose-response curve showing inhibition of OLP induction by SHHAg in the presence of increasing concentrations of PD173074 at DIV4. Half-maximal inhibition occurs at ~25 nM PD173074, as described for inhibition of FGFR1 itself (Dimitroff et al., 1999).

inducing activity of FGF2 in dorsal spinal cord cultures (Fig. 1F). In contrast, the OLIG2-inducing activity of SHHAg in cortical cultures was strongly inhibited by both cyclopamine (Fig. 4B) and PD173074 (Fig. 4A,B). We also looked at induction of NG2-positive OLPs at DIV4, with analogous results, i.e. NG2-induction by FGF was blocked by PD173074 (not shown) but not by cyclopamine (Fig. 4A), whereas induction by SHHAg was sensitive to both reagents (Fig. 4A and not shown). The concentration of PD173074 required for half-maximal inhibition of SHHAg was ~25 nM, similar to that reported for FGFR1 itself (Dimitroff et al., 1999) (Fig. 4C). We also determined that PD173074 does not inhibit the closely related PDGFR (see Materials and methods). Our data suggest that SHH ultimately relies on activation of FGFR, either directly or indirectly, for its OLIG2- and OLP-inducing abilities.

We tried to determine whether FGFR activation in SHH-treated cells requires extracellular FGF, by sequestering FGF outside cells with recombinant, extracellular fragments of FGFR1. We used a mixture of FGFR1αIIIc and FGFR1βIIIc alternative splice isoforms, which can bind FGF2 and other FGFR1-binding isoforms at high affinity. These reagents effectively prevented induction of OLIG2 by added FGF2 but had no effect on OLIG2 induction by SHHAg (Fig. 4B). Taken together, our data suggest that SHH activity requires ligand-independent activation of FGFR. Perhaps the G-protein-coupled SHH receptor SMO trans-activates FGFR inside cells. Alternatively, SHH might not itself trans-activate FGFR, but might rely on a basal level of FGFR activity that is constitutive in our cultures.

Induction of OLIG2 expression by SHHAg or FGF2 requires MAPK activity

If SHH and FGF both act through FGFR as implied above, one would expect them to trigger the same intracellular signalling pathways. FGFR activation leads to autophosphorylation of the TK domains, which in turn can initiate MAPK and pathways and elevation of intracellular calcium. We investigated the involvement of MAPK and PI 3-kinase signalling pathways, using synthetic drugs that inhibit MEK1/2 (U0126) or PI 3-kinase (LY294002).

We found that induction of OLIG2 by either FGF2 or SHHAg was strongly inhibited by U0126, but not by LY294002, at either DIV1 (Fig. 5A) or DIV2 (not shown), indicating that the MAPK pathway but not the PI 3-kinase pathway is crucial for this first step of lineage specification. We visualised MAPK activation directly by immunofluorescence microscopy with an antibody that specifically recognises the phosphorylated form of the protein. Within 1 hour of FGF2 exposure there was a marked increase in MAPK immunolabelling over control (compare Fig. 5Ba with Bk). Surprisingly (given the data of Fig. 5A), we could detect no increase in MAPK immunolabelling after SHHAg treatment (compare Fig. 5Ba and 5Bg).

We confirmed these findings by looking directly at p42/p44 (MAPK) activation by western blotting with an antibody directed against the phosphorylated forms of p42/p44 (Fig. 5C). As expected, FGF2 caused a large increase in the level of MAPK phosphorylation within 1 hour (compare lanes 1 and 3). SHH did not cause significant MAPK activation at one hour (compare lanes 1 and 2). After 18 hours incubation with FGF2 there was a small residual increase in phosphorylated MAPK compared with control (compare lanes 4 and 7). However SHH still had no effect on MAPK (lanes 4, 5).

FGFR maintains a constitutive low level of active MAPK that is required for SHH activity

The inability of SHHAg to activate MAPK argues against trans-activation of FGFR, since direct stimulation by FGF2 causes robust MAPK activation. What, then, is the essential

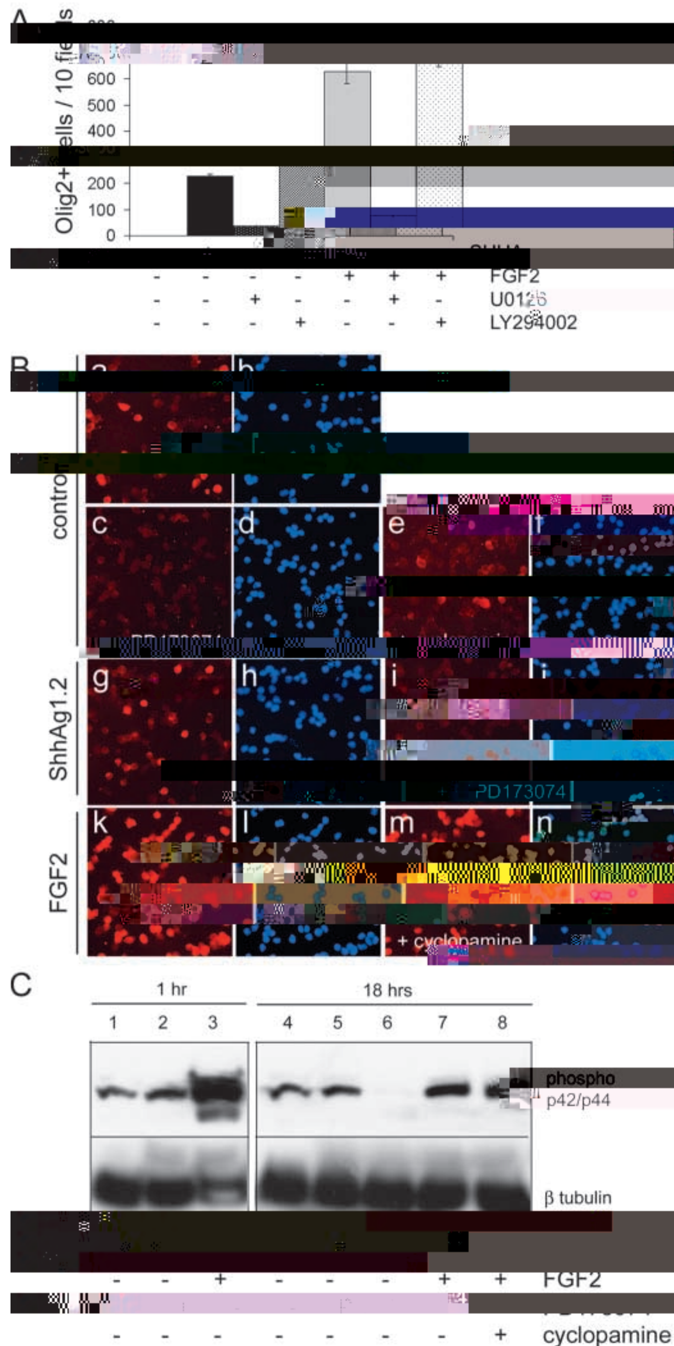

Fig. 5. OLP induction by SHH depends on activation of the MAPK pathway by FGFR1. (A) Neocortical neuroepithelial cells cultured for 24 hours in the presence of the MEK1/2 inhibitor U0126 and either SHHAg or FGF2 fail to develop Olig2-positive cells. The inhibitor of PI 3-kinase, LY294002, has no effect on the inducing activities of either SHHAg or FGF2. (B) To assess whether FGF2 and/or SHH activate the MAPK pathway we cultured E13.5 cortical cells in the absence (a-f) or presence of either SHHAg (g-j) or FGF2 (k-n), together with PD173074 (c,i) or cyclopamine (e,m) for 1 hour prior to immunolabelling with an anti-phospho-ERK1/2 antibody and Hoechst dye (b,d,f,h,j,l,n). FGF2 by itself caused strong activation of MAPK. SHH failed to activate MAPK above endogenous levels (compare a, g) and all MAPK activity was abolished by PD173074 (c,i). (C) Protein lysates from cortical cultures incubated with FGF2 or SHHAg and PD173074 or cyclopamine for 1 hour or 18 hours were separated by PAGE, and analysed for the presence of phosphorylated ERK1/2 (p42/p44) by western blot. SHHAg failed to activate MAPK above control levels, and PD173074 abolished all

role of FGFR in the activity of SHH? In the absence of added SHH or FGF2 there is a background of active MAPK in our cultures (Fig. 5Ba and 5C lanes 1, 4), but this background is abolished by adding PD173074 (Fig. 5Bc). Even in the presence of SHH, the basal level of active MAPK is obliterated by PD173074 (Fig. 5Bi, and lane 6 in C). Therefore, it seems likely that the steady-state level of active MAPK in our cultures is caused by low, constitutive FGFR activity and that this basal activity is absolutely required for OLIG2 induction by SHH.

Cell-autonomous requirement for MAPK activation in SHH-responding cells

The experiments described above showed that MAPK phosphorylation is required in neocortical cultures for the OLP-inducing activity of SHH but did not distinguish between a direct or indirect effect of MAPK. For example, MAPK might stimulate release of a diffusible factor that acts secondarily on neighbouring cells to render them responsive to SHH. Alternatively, MAPK might be required within the same cells that respond to SHH.

We addressed this question by infecting neocortical precursors with a retrovirus vector encoding a mutated form of RAS that constitutively activates the MAPK pathway. The retrovirus also encodes the enhanced green fluorescent protein (eGFP) so that infected cells can be positively identified using the fluorescence microscope. Unsurprisingly, we found that constitutively active RAS was not by itself sufficient to activate OLIG2 expression in the absence of SHH signalling (added cyclopamine; Fig. 6Ab-d). However, in the presence of SHHAg and PD173074 (to block MAPK activation via FGFR) the only cells that expressed OLIG2 were those that also expressed activated RAS (Fig. 6Af-g,B). Note that not all cells that expressed active RAS also expressed OLIG2 (Fig. 6Af-h). These observations allow us to conclude, (1) the MAPK pathway is necessary but not sufficient for OLIG2 induction as SHH signalling is also required, and (2) MAPK activation is required cell-autonomously, i.e. it acts directly in the SHH-targeted cells.

Two stages of OPC induction (OLIG2, NG2) with different signalling requirements

We investigated the requirement for MAPK and PI 3-kinase in the later transition from OLIG2-positive, NG2-negative ($OLIG2^+$, $NG2^–$) to ($OLIG2^+$, $NG2^+$) OLPs. We first allowed ($OLIG2^+$, $NG2^–$) cells to develop until DIV2 under the influence of FGF2 or SHHAg, then added the MAPK and/or PI 3-kinase inhibitors for a further 2 days (until DIV4) before immunolabelling with anti-NG2. We found that NG2 expression was inhibited strongly by both drugs (Fig. 7), indicating that both the MAPK and PI 3-kinase pathways are important during this later stage of oligodendrocyte lineage progression. Thus, there are different signalling requirements for the initial specification event (MAPK only) compared to later lineage progression (MAPK and PI 3-kinase).

FGFR signalling is not required for SHH activity in the ventral spinal cord or forebrain

The data described above raised the possibility that induction of OLPs in the ventral neural tube in vivo, which is known to

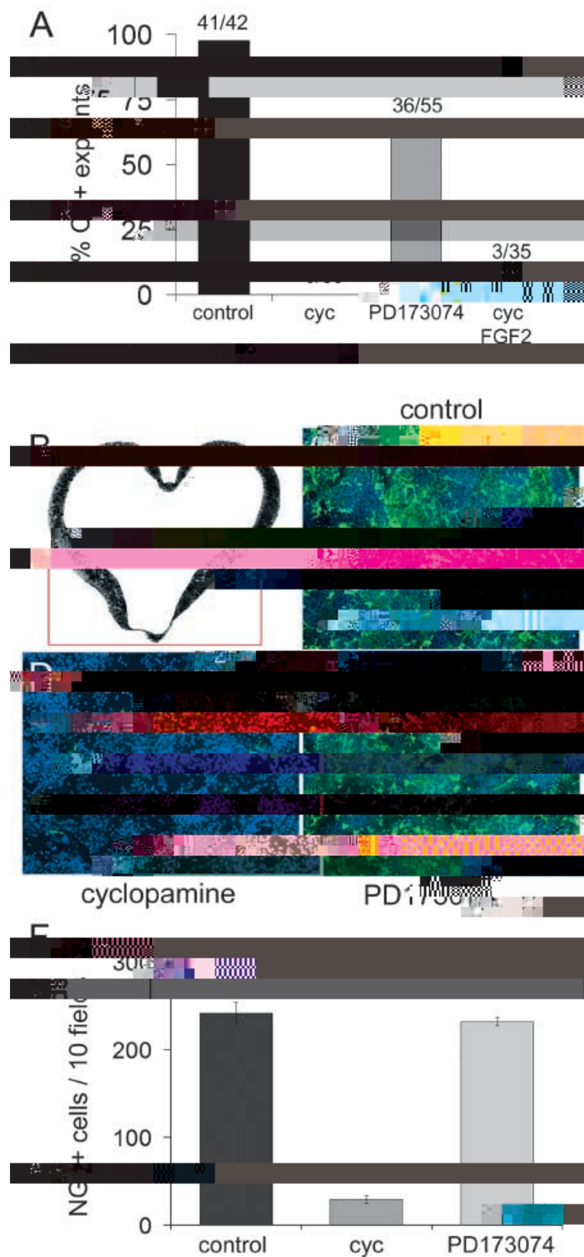

Fig. 8. Induction of OLPs in ventral spinal cord or ventral forebrain cultures is independent of FGFR-TK activity. (A) Ventral spinal cord explants from Hamilton and Hamburger stage 12 (E2) chicks developed O4-positive OLPs when cultured without exogenously added growth factors. Their development was strongly inhibited by

or FGF2. This inherent potential takes a long time to manifest itself (DIV6) and can be blocked by cyclopamine, implying that endogenous Hedgehog activity in the cultures is largely responsible (Tekki-Kessaris et al., 2001). Consistent with this, we found that mRNAs encoding SHH and its relative Indian Hedgehog (IHH) were up-regulated in the cultures (Tekki-Kessaris et al., 2001). Recently, Gabay et al. (Gabay et al., 2003) reported that neocortical cells in monolayer or neurosphere culture up-regulate SHH in response to FGF2 (0.2 ng/ml) and that the OLIG2-inducing activity of this low concentration of FGF2 can be blocked by cyclopamine. This suggests that the up-regulation of Hedgehog transcripts that we observed previously (Tekki-Kessaris et al., 2001) might be due to endogenous FGFR activation and that part of the OLP-inducing activity of added FGF might be mediated indirectly through Hedgehog proteins. However, that cannot account for all of the effect of FGF when added at the higher concentrations (10 ng/ml) used in our present study, because in our hands FGF-mediated OLP induction was not inhibited significantly by cyclopamine. On the contrary, we found that the OLP-inducing activity of SHHAg is dependent on FGFR and MAPK. However, MAPK alone is not sufficient to induce OLPs – SHH signalling is also required. The additional obligatory signal that is triggered by SHH is presumably also triggered by FGFR, since FGF2 can induce OLPs independently of SHH.

Despite its critical role in cortical precursors, we found that FGFR is not required for SHH-mediated cell fate specification in ventral spinal cord or forebrain, even though ventral precursors are known to express FGFR1-3 in vivo. It is possible that receptor TKs other than FGFR collaborate with SHH in non-cortical cells.

Do FGF2 and SHH act on the same population of cortical precursors?

FGF2 activated the MAPK pathway rapidly in all, or nearly all, E13 cortical precursors (Fig. 5Bk). This is consistent with the fact that FGFR1-3 are expressed in most cortical cells at this age. However, only a minority of the MAPK-active cells – around 10% – went on to express OLIG2 at DIV2 (not shown). A similar proportion of precursors expressed OLIG2 after SHHAg stimulation. What distinguishes the precursor cells that are competent to express OLIG2 from their OLIG2-incompetent neighbours is a mystery.

The FGF2 and SHH-responsive cells could belong to the same or different populations. The simplest interpretation of our data – the one we prefer – is that SHH and FGF2 act directly on the same sub-population of cortical precursors to activate OLIG2. This interpretation is strengthened by our finding that MAPK and SHH act together in the same precursors.

Is FGF involved in oligodendrocyte generation in vivo?

We and others have presented evidence that OLPs are generated in the ventral spinal cord and forebrain during embryogenesis and migrate from there into more dorsal territories including the cerebral cortex (Warf et al., 1991; Pringle and Richardson, 1993; Noll and Miller, 1993; Timsit et al., 1995; Spassky et al., 1998; Nery et al., 2001; Tekki-Kessaris et al., 2001). Chick-

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