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Fgfr3 expression by astrocytes and their precursors: evidence that astrocytes and oligodendrocytes originate in distinct neuroepithelial domains

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SUMMARY

The postnatal central nervous system (CNS) contains many scattered cells that express fibroblast growth factor receptor 3 transcripts (*Fgfr3*). They first appear in the ventricular zone (VZ) of the embryonic spinal cord in mid-gestation and then distribute into both grey and white matter – suggesting that they are glial cells, not neurones. The *Fgfr3*$^+$ cells are interspersed with but distinct from platelet-derived growth factor receptor α (*Pdgfra*)-positive oligodendrocyte progenitors. This fits with the observation that *Fgfr3* expression is preferentially excluded from the pMN domain of the ventral VZ where *Pdgfra*$^+$ oligodendrocyte progenitors – and motoneurones – originate. Many glial fibrillary acidic protein (Gfap)-positive astrocytes co-express *Fgfr3* in vitro and in vivo. *Fgfr3*$^+$ cells within and outside the VZ also express the astroglial marker glutamine synthetase (*Gln*s). We conclude that (1) *Fgfr3* marks astrocytes and their neuroepithelial precursors in the developing CNS and (2) astrocytes and oligodendrocytes originate in complementary domains of the VZ. Production of astrocytes from cultured neuroepithelial cells is hedgehog independent, whereas oligodendrocyte development requires hedgehog signalling, adding further support to the idea that astrocytes and oligodendrocytes can develop independently. In addition, we found that mice with a targeted deletion in the *Fgfr3* locus strongly upregulate Gfap in grey matter (protoplasmic) astrocytes, implying that signalling through Fgfr3 normally represses Gfap expression in vivo.

Key words: Fgfr3, Targeted deletion, Astrocyte, Reactive gliosis, CNS, Neuroepithelium

INTRODUCTION

In the embryonic CNS, neurones and glia develop from the neuroepithelial cells of the ventricular zone (VZ) that surrounds the ventricles of the brain and the lumen of the spinal cord. Different domains of the VZ express different gene products and generate different subsets of neurones and/or glia. For example, the ventral half of the spinal cord VZ is subdivided into five regions labelled (from ventral to dorsal) p3, pMN, p2, p1 and p0. These five domains express different combinations of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors and generate distinct classes of spinal neurones; pMN gives rise to somatic motoneurones, whereas p0-p3 give rise to four classes of ventral interneurones (V0-V3 respectively) (reviewed by Briscoe and Ericson, 1999; Jessell, 2001). In the brainstem, p3 also gives rise to visceral motoneurones (Ericson et al., 1997).

After neurones, the VZ switches to producing glial cells. Oligodendrocytes, the myelinating glial cells of the CNS, develop from the ventral VZ. Small numbers of oligodendrocyte progenitors (OLPs), which express the platelet-derived growth factor receptor-α (Pdgfra), first appear at the ventricular surface on embryonic day 12.5 (E12.5) in the mouse, then proliferate and migrate away into the grey and white matter before starting to differentiate into myelin-forming oligodendrocytes (Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). In rodents, OLPs are generated from the same part of the neuroepithelium as somatic motoneurones (MNs) but not until after MN production has ceased (Sun et al., 1998; Lu et al., 2000) (for a review, see Rowitch et al., 2002). This prompted us to suggest that there is a pool of shared neuroglial precursors that first generates MNs, then switches to OLPs (Richardson et al., 1997; Richardson et al., 2000). This idea has been supported recently by the finding that the bHLH proteins Olig1 and Olig2 are expressed and required in pMN for production of both motoneurones and OLPs (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002) (reviewed by Rowitch et al., 2002).

Where do astrocytes, the other major class of CNS glia, originate in the neuroepithelium? It is believed that at least some astrocytes are generated by transdifferentiation of radial

glia (Bignami and Dahl, 1974; Choi et al., 1983; Benjelloun-Touimi et al., 1985; Voigt, 1989; Culican et al., 1990). Others are formed from multipotent precursors in the subventricular zones (SVZ) of the postnatal brain. However, the origins of astrocytes in the developing spinal cord are unclear, so we looked for an astrocyte lineage marker that might be helpful in following the development of astrocytes from their earliest precursors in the VZ. Previous expression studies of the fibroblast growth factor receptor 3 (*Fgfr3*) suggested that this receptor might be expressed in glial cells, possibly astrocytes (Peters et al., 1993; Miyake et al., 1996). Our own studies, reported here, support this conclusion and suggest that *Fgfr3*-positive astrocytes develop from *Fgfr3*-positive precursor cells in the neuroepithelium. *Fgfr3* is not expressed equally in all parts of the neuroepithelium but is reduced or absent from pMN, suggesting that astrocytes and OLPs have separate neuroepithelial origins. We also found that astrocytes are formed in vitro in the absence of hedgehog signalling – unlike oligodendrocytes, which require sonic hedgehog from the ventral midline. This reinforces the notion that at least some astrocytes develop independently of OLPs.

To investigate the function of *Fgfr3* in astrocytes, we examined mice with a targeted deletion in the *Fgfr3* locus (Colvin et al., 1996). The number of *Fgfr3*-expressing cells was normal in the knockout, suggesting that *Fgfr3* does not mediate a mitogenic or survival-promoting effect for these cells. However, Gfap was markedly upregulated in grey matter astrocytes, which normally have little or no Gfap – unlike their counterparts in white matter. Our results imply that signalling through Fgfr3 normally represses Gfap expression in grey matter astrocytes and suggest that white matter astrocytes might preferentially express Gfap because ligands for Fgfr3 are not normally available in axon tracts.

MATERIALS AND METHODS

Tissue and cell cultures

Spinal cords from stage 12-13 (48 hour) chick embryos were dissected into dorsal, middle and ventral thirds using a flame-sharpened tungsten needle. Tissue fragments were cultured as explants in collagen gels (Guthrie and Lumsden, 1994) in defined BS medium (Bottenstein and Sato, 1979) containing 0.25% (v/v) foetal bovine serum (FBS) and conalbumin in place of transferrin (Pringle et al., 1996).

For dissociated cell cultures, E17 rat cervical spinal cords were digested in 0.25% (w/v) trypsin in Earle's buffered saline (Ca^{2+} and Mg^{2+} free; Gibco) for 15 minutes at 37°C, then FBS was added to a final concentration of 10% (v/v) and the tissue physically dissociated by trituration. Cells were washed by centrifugation and resuspended in BS medium before plating in a 50 μl droplet on poly-D-lysine-coated glass coverslips (5×10^4 cells/coverslip). Both explants and dissociated cell cultures were cultured at 37°C in 5% CO_2 in a humidified atmosphere.

Neutralising Shh activity in vitro

Monoclonal Shh neutralising antibody 5E1 (Ericson et al., 1996) was concentrated by ammonium sulphate precipitation from hybridoma supernatants (Harlow and Lane, 1988). Monoclonal anti-NG2 proteoglycan #4.11 (Stallcup and Beasley, 1987) was used as a negative control. Precipitated antibodies were dissolved in a small volume of PBS and dialysed first against PBS and then Dulbecco's modified Eagle's medium (DMEM, Gibco). The final volumes were approximately tenfold less than the starting volumes and were assumed to be ten time as concentrated. Explants were incubated in the presence of either anti-Shh or control antibodies at twice the final concentration. Antibodies were added at the start of the experiment and fresh medium and antibody were added each day thereafter. In some experiments cyclopamine (1 μM; from William Gaffield) instead of anti-Shh was added to cultures daily.

BrdU labelling in vivo

E18 pregnant mice were injected intraperitoneally with BrdU at 50 μg BrdU per gram bodyweight. Two injections were given, 6 hours apart. Mice were sacrificed 3 hours after the second injection and the embryos were processed for *Fgfr3* in situ hybridisation and BrdU immunolabelling.

Preparation of tissue sections

C57Bl/6 mice were obtained from Olac and bred in-house. Noon on the day of discovery of the vaginal plug was designated embryonic day 0.5 (E0.5). We also used *Fgfr3*-null mice (Colvin et al., 1996) bred at UCL. Mid-gestation embryos were staged according to the morphological criteria of Theiler (Theiler, 1972). Rats (Sprague-Dawley) were obtained from the UCL breeding colony and staged according to Long and Burlingame (Long and Burlingame, 1938). Fertilised White Leghorn chicken eggs were obtained from Needle Farm (Cambridge, UK). They were incubated at 38°C and the chicken embryos staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Embryos were decapitated and immersion-fixed in cold 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours before cryoprotecting in cold 20% (w/v) sucrose in PBS for at least 24 hours. In sections processed for immunohistochemistry after in situ hybridisation, the fixation time was reduced to 1 hour to preserve epitope integrity. Tissues were immersed in OCT embedding compound (BDH), frozen on solid CO_2 and stored at –70°C before sectioning. Frozen sections (15 μm) were cut on a cryostat and collected on 3-aminopropyl-triethoxysilane (APES)-coated glass microscope slides. Sections were air-dried for 2 hours before storing dry at –70°C.

Immunohistochemistry

Anti-Gfap monoclonal ascites, clone G-A-5 (Sigma), was used at a dilution of 1:400. Anti-BrdU (monoclonal BU209) (Magaud et al., 1989) was used at 1:5 dilution. Monoclonal O4 (Sommer and Schachner, 1981) was used as cell culture supernatant diluted 1:5. Secondary antibodies were rhodamine- or fluorescein-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin (all from Pierce) diluted 1:200. All antibodies were diluted in PBS containing 0.1% (v/v) Triton X-100 and 10% (v/v) normal goat serum, except O4, which was diluted in PBS alone. Sometimes diaminobenzidine (DAB) labelling (ABC kit, Vector Laboratories) was used instead of fluorescence detection.

In situ hybridisation

Our in situ hybridisation procedures have been described (Pringle et al., 1996; Fruttiger et al., 1999); detailed protocols are available at http://www.ucl.ac.uk/~ucbzwdr/richardson.htm. Digoxigenin (DIG)- or fluorescein (FITC)-labelled RNA probes were transcribed in vitro from cloned cDNAs. The rat *Fgfr3* probe was transcribed from a ~900 bp partial cDNA encoding most of the tyrosine kinase (TK) domain (W.-P. Yu, PhD thesis, University of London, 1995) and the chicken *Fgfr3* probe from a ~440 bp partial cDNA encoding part of the TK domain (from Ivor Mason, King's College London). The mouse *Pdgfra* probe was made from a ~1600 bp cDNA encoding most of the extracellular domain_(from Chiayeng Wang, University of Chicago). The chicken *Pdgfra* probe was made from a ~3200 bp cDNA covering most of the 3′ untranslated region of the mRNA (from Marc Mercola, Harvard Medical School, Boston).

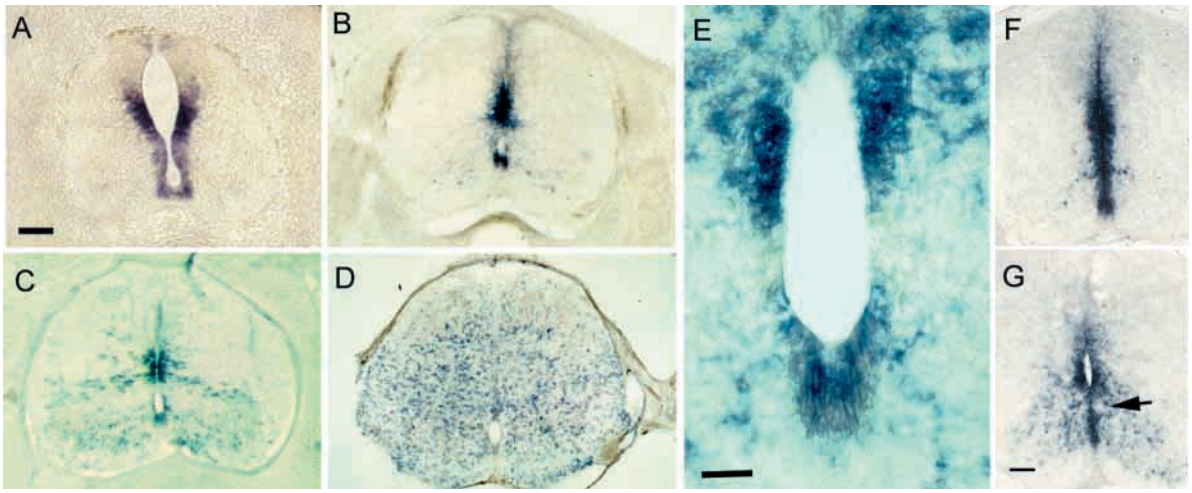

Fig. 1. *Fgfr3* expression in transverse sections of embryonic chick and mouse cervical spinal cords. (A) Chick stage 22-24 (E3.5-4); (B) chick stage 34 (E8); (C) chick stage 35 (E9); (D) chick stage 37 (E11); (E) chick stage 35 (E9); (F) mouse E13.5; and (G) mouse E14.5. Initially, *Fgfr3* is expressed in the floor plate and the ventral two-thirds of the VZ (A) and is later downregulated in part of the ventral VZ (B). Starting around stage 34 (E8) *Fgfr3*$^+$ cells are visible in the parenchyma of the cord. By stage 37 (E11) the floor plate and VZ no longer express *Fgfr3* but scattered *Fgfr3*$^+$ cells are present throughout most of the cross-section of the cord, including both grey and white matter (D). (E) A magnified image of the ventral VZ from a stage 35 (E9) cord, showing the two spatially separated domains of *Fgfr3* expression. A similar progression occurs in mouse (F,G). However, the ventral 'gap' is not so pronounced in mouse (arrow in G). Scale bars: 200 μm (A-D), 100 μm (F,G), 50 μm (E).

For double in situ hybridisation, two probes – one FITC labelled and the other DIG labelled – were applied to sections simultaneously. The FITC signal was visualised with alkaline phosphatase (AP)-conjugated anti-FITC Fab_2 fragments before developing in p-iodonitrotetrazolium violet (INT) and 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) (BCIP), which produces a magenta/brown reaction product. The sections were photographed, then the AP was inactivated by heating at 65°C for 30 minutes followed by incubating in 0.2 M glycine (pH 2) for 30 minutes at room temperature. The INT-BCIP reaction product was removed by dehydration through graded alcohols, concluding with 100% ethanol for 10 minutes at room temperature. The DIG signal was then visualised with AP-conjugated anti-DIG Fab_2 fragments and a mixture of nitroblue tetrazolium (NBT) and BCIP (all reagents from Roche Molecular Biochemicals) and the sections re-photographed. No labelling with NBT/BCIP was observed when we omitted either the DIG labelled probe or the anti-DIG antibody (data not shown).

For the *Fgfr3-Pdgfra* double in situ hybridisation of Fig. 4 we visualised the FITC (*Pdgfra*) signal with horseradish peroxidase (HRP)-conjugated anti-FITC Fab_2 fragments (Roche) before developing in fluorescein-tyramide reagent (NEN^{TM} Life Science Products, Boston) according to the manufacturer's instructions. The HRP-conjugate was inactivated by incubating in 2% (v/v) hydrogen peroxide for 30 minutes at room temperature. The DIG (*Fgfr3*) signal was then visualised with HRP-conjugated anti-DIG Fab_2 fragments followed by rhodamine-tyramide, and the sections photographed under fluorescence optics. As specificity controls we omitted either the FITC-labelled *Pdgfra* probe or the HRP-conjugated anti-FITC antibody, which gave no staining other than for *Fgfr3* (not shown).

Combined immunolabelling and in situ hybridisation

For the experiment of Fig. 7, cultured cells were first subjected to in situ hybridisation with a [^{35}S]-labelled RNA probe against *Fgfr3* then immunolabeled with anti-Gfap and biotinylated goat-anti-mouse Ig. The Gfap signal was developed with DAB and the slides dehydrated through ascending alcohols, dipped in nuclear emulsion (Ilford K5), exposed in the dark for several days and developed in Kodak D19.

RESULTS

Fgfr3 expression in the embryonic spinal cord

We examined *Fgfr3* expression in the embryonic chick spinal cord by in situ hybridisation. At stage 22-24 (corresponding to ~E4), *Fgfr3* expression was confined to the floor plate and the ventral two-thirds of the VZ (Fig. 1A). By stage 34 (E8) *Fgfr3* expression had been extinguished in part of the ventral VZ so that a gap developed in the expression pattern (e.g. Fig. 1B).

Individual *Fgfr3*$^+$ cells were also present outside the VZ

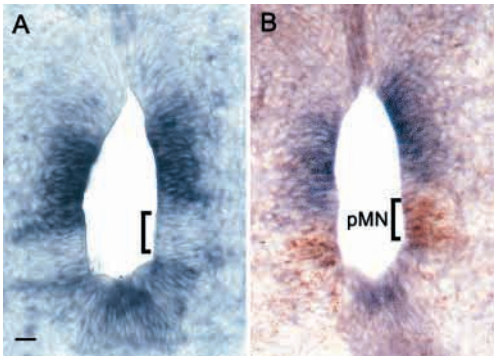

Fig. 2. Expression of *Fgfr3* and *Olig2*. Transverse sections through stage 35 (E9) chicken spinal cords were subjected to in situ hybridisation for *Fgfr3* (A) or double in situ for *Fgfr3* and *Olig2* (B). At this age, *Fgfr3* expression is confined to the VZ and a few scattered cells outside the VZ. The two spatially separated domains of *Fgfr3* expression are clearly visible (A). *Olig2* is expressed predominantly within the ventral 'gap' of *Fgfr3* expression (B). This suggests that pMN (brackets), which generates *Pdgfra*$^+$ oligodendrocyte progenitors (OLPs), does not also generate *Fgfr3*$^+$ putative astrocyte progenitors. Scale bar: 50 μm.

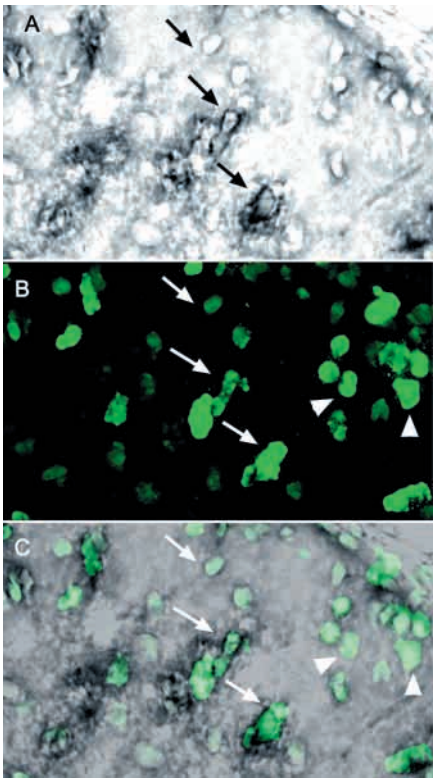

Fig. 3. Incorporation of BrdU by *Fgfr3*-expressing cells. We labelled E18 embryos by two intra-peritoneal injections of BrdU, 6 hours apart, into the mother. We harvested the embryos 3 hours later and performed in situ hybridisation for *Fgfr3* followed by immunohistochemistry for BrdU. The *Fgfr3* (A) and BrdU (B) images were superimposed using Adobe Photoshop (C). Many *Fgfr3*-expressing cells incorporated BrdU (C, arrows), confirming that they can divide after exiting the VZ and are therefore unlikely to be neurones. Arrowheads in B,C indicate *Fgfr3*-negative cells that have incorporated BrdU.

after stage 34 (E8), both lateral and dorsal to the *Fgfr3*$^+$ neuroepithelial domains. Often the individual cells appeared to be streaming away from the VZ into the parenchyma. This is evident in Fig. 1C, for example. By stage 37 (E11) *Fgfr3* expression was no longer detectable in the VZ but scattered *Fgfr3*$^+$ cells were present throughout the grey and white matter of the cord (Fig. 1D). *Fgfr3* expression followed a similar progression in mouse and rat (Fig. 1F,G and not shown). In rodents, however, the ventral gap in *Fgfr3* expression was not as pronounced as in chicks (Fig. 1G, arrow).

A scattered population of *Fgfr3*-expressing cells is found throughout most regions of the late embryonic and postnatal mouse brain, both in white and in grey matter. As in the embryonic spinal cord, there appear to be specific regions of the embryonic brain VZ that give rise to *Fgfr3*$^+$ cells that stream away from the VZ into the parenchyma (not shown).

Fgfr3-expressing cells originate mainly outside the pMN domain of the neuroepithelium

In the developing spinal cord, neuroepithelial precursors at different positions along the dorsoventral axis generate distinct neuronal subtypes. The ventral half of the spinal cord VZ is

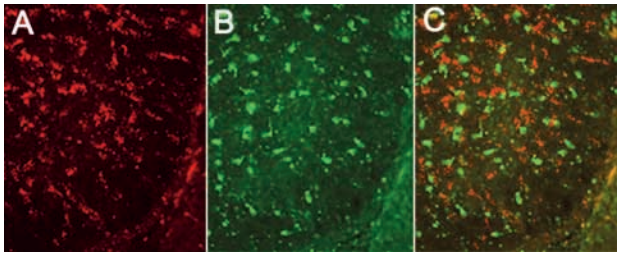

Fig. 4. Different populations of *Fgfr3*$^+$ and *Pdgfra*$^+$ cells in the newborn spinal cord. We hybridised sections of P2 mouse cervical spinal cord simultaneously with a DIG-labelled *Fgfr3* probe together with an FITC-labelled *Pdgfra* probe to visualise OLPs. The *Fgfr3* signal (red) was visualised with rhodamine-tyramide reagent and the *Pdgfra* signal (green) with fluorescein-tyramide. Scattered individual *Fgfr3*$^+$ and *Pdgfra*$^+$ cells can be seen throughout both white and grey matter of the cord, but these are separate and discrete cell populations. We conclude that the great majority of *Fgfr3*$^+$ cells in the cord are not OLPs.

divided into five neuroepithelial domains known as (from ventral to dorsal) p3, pMN, p2, p1 and p0 (Briscoe et al., 2000). Of these, pMN is known to generate motoneurones followed by oligodendrocyte progenitors (OLPs). It seemed to us that the ventral gap in *Fgfr3* expression (Fig. 2A) might correspond to pMN. To test this, we performed double in situ hybridisation for *Fgfr3* and *Olig2* (which defines pMN) (Lu et al., 2000; Zhou et al., 2000). At stage 35, the *Olig2* in situ hybridisation signal was within the gap in the *Fgfr3* signal (Fig. 2B, arrow). Therefore, *Fgfr3* is preferentially downregulated in pMN where oligodendrocyte lineage cells originate, but is expressed both ventral and dorsal to pMN.

Fgfr3- expressing cells are glia

The fact that most of the scattered *Fgfr3*$^+$ cells are generated after stage 34 (E8) in the chick, E13.5 in mouse, is itself a strong argument that they are glial cells, not neurones, because most spinal neurones are born before this (Altman and Bayer, 1984). That some of the *Fgfr3*$^+$ cells are found in axon tracts also suggests that they are glia, for there are very few neuronal cell bodies in fibre tracts.

Another indication that they are glial cells is that they continue to divide after they leave the VZ. We showed this by injecting BrdU into a pregnant mouse at18 days gestation. The embryos were removed 3 hours later and processed by in situ hybridisation for *Fgfr3* followed by immunolabelling for BrdU. We found many (*Fgfr3*$^+$, BrdU$^+$) cells scattered throughout the white and grey matter of the cord (Fig. 3, arrows). This confirms that *Fgfr3*-expressing cells divide in vivo and are therefore unlikely to be neurones or neuronal progenitors, which leave the VZ as postmitotic cells. This strengthens the idea that the *Fgfr3*-expressing cells are glia. There was also a population of (BrdU$^+$, *Fgfr3*$^-$) cells in both grey and white matter (Fig. 3C, arrowheads), so there is a distinct population(s) of dividing cells that do not express *Fgfr3*.

Fgfr3-expressing cells are distinct from Pdgfra$^+$ oligodendrocyte progenitors

To determine whether the *Fgfr3*$^+$ cells that we detect are oligodendrocyte progenitors (OLPs), we double labelled

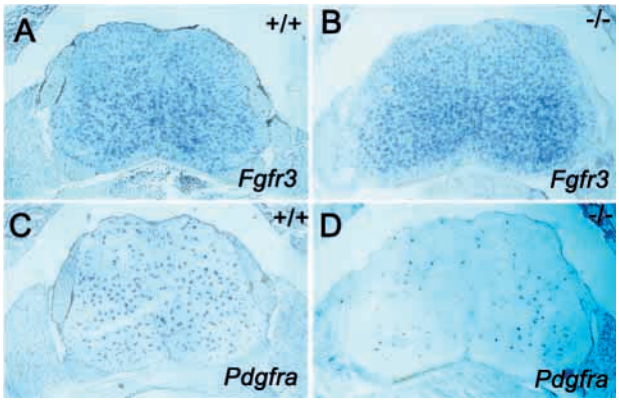

Fig. 5. *Fgfr3*-positive cells are unaffected in *Pdgfa* null spinal cords. Consecutive sections of newborn wild-type or *Pdgfa* knockout mouse cervical spinal cords were hybridised in situ with probes to *Fgfr3* (A,B) or *Pdgfra* (C,D). The number of *Pdgfra*$^+$ OLPs is strongly reduced in the *Pdgf-A* knockout (compare C with D) but neither the number nor the distribution of *Fgfr3*$^+$ cells is changed noticeably (A,B). Again, we conclude that the *Fgfr3*$^+$ cells and *Pdgfra*$^+$ OLPs are different cells.

mouse E18 and P2 spinal cord sections for *Fgfr3* and *Pdgfra*, an established marker of early OLPs. Both in situ hybridisation probes labelled similar numbers of cells that were scattered throughout the spinal cord grey and white matter, but the two cell populations were completely non-overlapping (Fig. 4). This also held true throughout the postnatal brain (N. P. P., unpublished). We also looked in newborn *Pdgfa* knockout mice which contain far fewer *Pdgfra*$^+$ OLPs than normal (Fruttiger et al., 1999). Despite the lack of OLPs, there were normal numbers of *Fgfr3*$^+$ cells at this age (Fig. 5). Clearly, the *Fgfr3*$^+$ cells detected by our in situ hybridisation procedures are not early OLPs but a different cell population. This is consistent with the fact that in mice lacking Fgfr3, early events of oligodendrocyte lineage progression occur normally and the numbers of *Pdgfra*$^+$ cells remains unchanged (R. Bansal, personal communication) (N. P. P., unpublished).

Fgfr3-expressing cells are astrocytes and astrocyte precursors

To test whether the *Fgfr3*$^+$ cells might be astrocytes, we double labelled E18 mouse spinal cord sections for *Fgfr3* and *Gfap* mRNAs. At E18, white matter astrocytes begin to express *Gfap* mRNA, which initially remains in the astrocyte cell bodies and allows identification of individual astrocytes. (As astrocytes mature further, both *Gfap* mRNA and protein are relocated to the extending cell processes, making individual cells difficult to distinguish.)

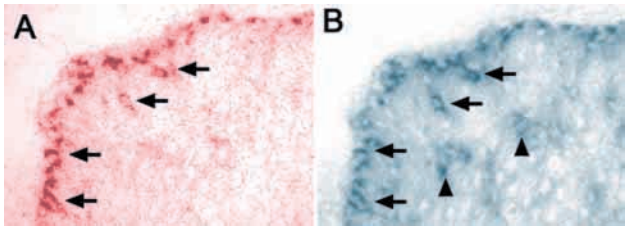

Fig. 6. Newly differentiating white matter astrocytes express *Fgfr3*. We simultaneously hybridised sections of E18 mouse cervical spinal cord with an FITC-labelled *Gfap* mRNA probe (A) and a DIG-labelled *Fgfr3* probe (B). The *Gfap* and *Fgfr3* hybridisation signals were visualised and photographed sequentially (see Materials and Methods). All the *Gfap*-expressing astrocytes also expressed *Fgfr3* (e.g. arrows). In general, *Fgfr3*$^+$ cells in the grey matter (arrowheads) did not co-express *Gfap*.

All the *Gfap*$^+$ astrocytes in developing white matter at E18 also expressed *Fgfr3* (Fig. 6, arrows). This result clearly identifies many of the *Fgfr3*-expressing cells as astrocytes. Nevertheless, the majority of *Fgfr3*-expressing cells in the grey matter (Fig. 6B, arrowheads) are *Gfap*-negative. We presume that these represent *Gfap*-negative, possibly immature, astrocytes.

In an attempt to label all astrocytes, including *Gfap*-negative astrocytes, we used an in situ hybridisation probe against glutamine synthetase mRNA (*Glns*) (EC 6.3.1.2). Glns is widely regarded as an astrocyte marker, although there have been reports that it is also present in mature oligodendrocytes and even OLPs. We found that *Glns* transcripts were present in the VZ of the E15 mouse spinal cord and in cells outside the VZ in a pattern that was very similar that of *Fgfr3* (Fig. 7). This is consistent with the view that *Fgfr3* and *Glns* mark astrocytes and their precursors. This conclusion was further strengthened by studies of cultured astrocytes (see below).

Cultured astrocytes co-express Gfap and Fgfr3

When CNS cells are dissociated and placed in culture, astrocytes in the culture upregulate Gfap and are easily recognisable. We dissociated and cultured cells from E17 rat cervical spinal cord and labelled them by in situ hybridisation for *Fgfr3* and by immunocytochemistry for Gfap. Almost all of the Gfap$^+$ astrocytes also expressed *Fgfr3* (Table 1; Fig. 8, arrows). There was also a small population of flat, fibroblast-like *Fgfr3*$^+$ cells that did not express Gfap (Fig. 8, arrowheads). The number of these cells decreased with time in culture; at 3 days they were 6% of all cells, by 9 days less than 1% (Table 1). These (*Fgfr3*$^+$, Gfap$^-$) cells might be astrocyte precursors or immature astrocytes that have not yet upregulated Gfap. In any

Table 1. E17 rat spinal cord cell cultures double labelled for *Fgfr3* and Gfap

Days in vitro	*Fgfr3*$^+$ Gfap$^-$ (astrocyte precursors?)	*Fgfr3*$^+$ Gfap$^+$ (astrocytes)	*Fgfr3*$^-$ Gfap$^+$ (astrocytes)	*Fgfr3*$^-$ Gfap$^-$ (other cells)
3	17/275 (6%)	69/275 (25%)	None	189/275 (68%)
6	23/596 (4%)	82/596 (14%)	2/596 (<1%)	489/596 (82%)
9	4/645 (<1%)	197/645 (31%)	1/645 (<1%)	443/645 (69%)

Dissociated cells from E17 rat spinal cord were cultured for 3, 6 or 9 days and then subjected to in situ hybridization for *Fgfr3* mRNA followed by immunohistochemistry for Gfap protein. We counted astrocytes (*Fgfr3*$^+$, Gfap$^+$ and *Fgfr3*$^-$, Gfap$^+$), putative astrocyte precursors (*Fgfr3*$^+$, Gfap$^-$) and other unidentified cells (*Fgfr3*$^-$, GFAP$^-$). The great majority of GFAP-expressing astrocytes also expressed *Fgfr3*. These data are from a single representative experiment (duplicate coverslips); comparable results were obtained in two additional independent experiments.

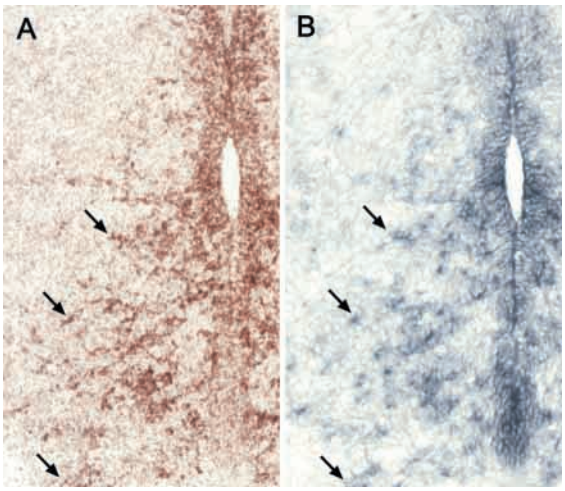

Fig. 7. Co-expression of *Fgfr3* and glutamine synthetase (*Glns*) in the VZ and parenchyma of the embryonic mouse spinal cord. There was considerable overlap between the in situ hybridisation signals for *Fgfr3* and *Glns* in the E15 mouse spinal cord, strongly suggesting that *Fgfr3*$^+$ cells correspond to glial (presumably astrocyte) precursors. Arrows indicate cells that express both *Fgfr3* and *Glns*.

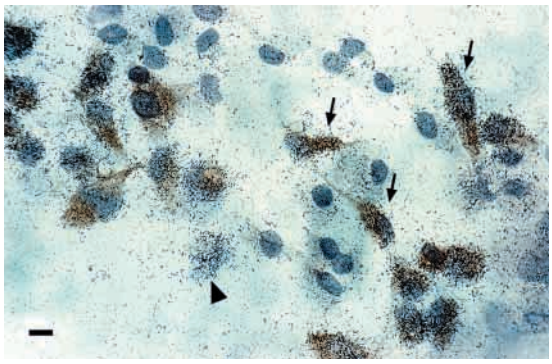

Fig. 8. Cultured cells from E17 rat spinal cord double-labelled for *Fgfr3* and Gfap. Cells were hybridised in situ with a ^{35}S-labelled RNA probe for *Fgfr3*, then immunolabelled for Gfap followed by autoradiography (see Materials and Methods). The *Fgfr3* signal (black silver grains) is present over most Gfap-positive cells (brown DAB reaction product; arrows) (also see Table 1). Scale bar: 10 μm. Arrowhead indicates an *Fgfr3*-positive, *Gfap*-negative cell.

case, this experiment provides clear evidence that most or all Gfap$^+$ astrocytes in culture co-express *Fgfr3*.

Gfap is upregulated in grey matter astrocytes in Fgfr3 null mice

If *Fgfr3* is expressed by astrocytes, we might expect to see specific effects on astrocytes in transgenic mice homozygous for a targeted disruption of *Fgfr3*. These mice have previously been shown to have skeletal and inner ear defects but no CNS defects have yet been reported (Colvin et al., 1996).

We visualised astrocytes in spinal cord sections of 3-month-old *Fgfr3* null mice, together with their heterozygous *Fgfr3*$^{+/-}$ and wild-type littermates, by immunolabelling with anti-Gfap. Heterozygous and null mutant mice all displayed the normal pattern of Gfap expression up to 6 weeks of age. Gfap expression was observed in the white matter around the circumference of the spinal cord, many Gfap-labelled processes being oriented in a radial direction (Fig. 9A). By comparison, there was little or no Gfap expression in the grey matter, except in astrocytes associated with blood vessels. Between 6 weeks and 2 months of age, a striking up-regulation of Gfap expression occurred in the grey matter of *Fgfr3* null mice, though not in their heterozygous or wild-type littermates (Fig. 9B). Astrocytes lining blood vessels also had increased Gfap immunoreactivity.

The number of cells that contain *Fgfr3* mRNA was not noticeably different in *Fgfr3*-null spinal cords compared with wild type (data not shown). This suggests that Fgfr3 does not normally mediate a signal for proliferation or survival of astrocytes, although further experiments (e.g. BrdU labelling in vivo) would be required to substantiate this.

Astrocyte development in vitro does not depend on Hedgehog signalling

In the spinal cord, production of ventral cell types – motoneurones, ventral interneurones and OLPs – is dependent on Shh signalling (Ericson et al., 1996; Orentas et al., 1999) (for a review, see Jessell, 2001). We wanted to know whether production of astrocytes from the ventral neural tube is also dependent on Shh. We microdissected stage 12/13 (E2) chick spinal cord into thirds along the dorsoventral axis and cultured the ventral-most fragments in collagen gels with either a control antibody or an anti-Shh neutralising antibody (see Materials and Methods). After 48 hours in culture we labelled explants with monoclonal antibody 4D5, which recognises homeodomain proteins Isl1 and Isl2 in motoneurones. Control explants contained numerous Isl$^+$ cells, whereas none were observed in explants incubated with anti-Shh (data not shown). After a further 10 days in culture (12 days total) we visualised OLPs with monoclonal antibody O4 (Sommer and Schachner, 1981) (Fig. 10C,D) and astrocytes with anti-Gfap (Fig. 10A,B). All of the explants incubated with control antibody (19/19) contained large numbers (>300) of O4$^+$ late-stage OLPs (Fig. 10C). As expected, OLP production was markedly decreased by anti-Shh (Fig. 10D); 14/22 explants contained no O4$^+$ cells and, of the remaining eight explants, seven contained fewer than ten positive cells and the other one contained 38 positive cells. By contrast, all explants contained numerous (>300) Gfap$^+$ astrocytes whether they had been incubated with control antibody (19/19) or anti-Shh (22/22) (Fig. 10A,B).

Similar results were obtained with explants from stage 25 (E5) embryos from which we were able to dissect the ventral one-quarter of the neural tube and discard the floor plate. Once again, large numbers of Gfap$^+$ astrocytes developed in explants cultured with control antibody (22/22) and with anti-Shh (25/25), even though OLP production in these explants was inhibited by anti-Shh (not shown).

To test the possibility that other hedgehog (Hh) proteins (Desert Hh, Indian Hh) control astrocyte production in ventral explants, we inhibited the activity of all isoforms with the alkaloid cyclopamine (Cooper et al., 1998; Incardona et al., 1998). This gave similar results as Shh neutralising antibodies (data not shown). Thus, we conclude that astrocyte induction in ventral spinal cord does not require Hedgehog signalling.

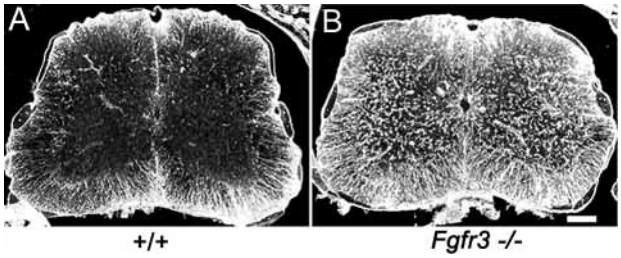

Fig. 9. Gfap upregulation in grey matter astrocytes in *Fgfr3*-null mice. Transverse sections through the cervical spinal cords of 2-month-old wild-type (A) and *Fgfr3*-null mice (B) were immunolabeled with anti-Gfap. In the wild-type cord, white matter (fibrous) astrocytes express Gfap but there is little or no Gfap immunoreactivity in the grey matter. By contrast, the *Fgfr3*-null mouse (B) shows extensive Gfap labelling of grey matter (protoplasmic) astrocytes. Scale bar: 100 μm.

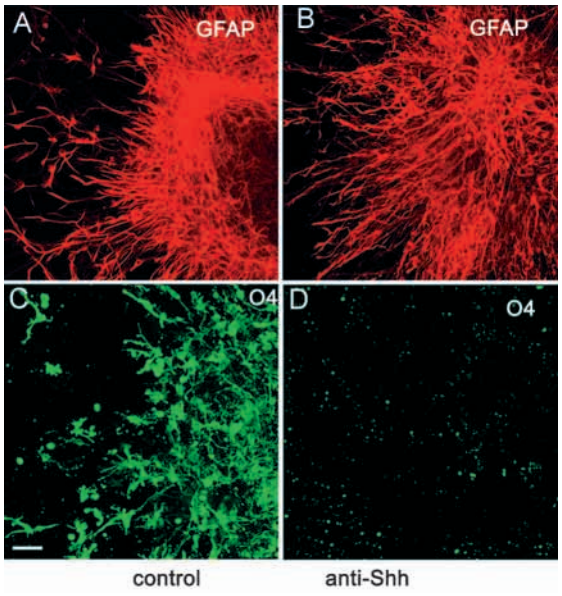

Fig. 10. Neutralising *Shh* activity in explant cultures of ventral spinal cord. Stage 12/13 (E2) chick neural tube was dissected into dorsal, intermediate and ventral thirds. The ventral thirds were cultured in collagen gels in the presence of either control antibody (A,C) or with neutralising anti-Shh antibody (B,D). Explants were double-labelled with O4 monoclonal antibody (C,D) and anti-Gfap (A,B). Anti-Shh blocks the formation of O4-positive OLPs but not Gfap-positive astrocytes. Scale bar: 10 μm.

DISCUSSION

On the basis of their spatial distribution and time of appearance, Peters et al. (Peters et al., 1993) suggested that *Fgfr3*$^+$ cells in the mouse CNS are glial cells, possibly astrocytes. By double labelling experiments with *Fgfr3* and Gfap, Miyake et al. (Miyake et al., 1996) concluded that *Fgfr3* was expressed in astrocytes in the adult rat brain. Our data support and extend these conclusions. We present evidence that scattered *Fgfr3*$^+$cells in the embryonic and postnatal CNS are astrocytes and/or astrocyte progenitors, and that these astrocytes are derived from *Fgfr3*$^+$ neuroepithelial precursors in the VZ.

Fgfr3 is also expressed transiently by a subpopulation of motoneurones (Philippe et al., 1998) and by late oligodendrocyte progenitors (late OLPs) just prior to terminal differentiation in vitro (Bansal et al., 1996). We are convinced that the *Fgfr3*$^+$ cells that we detect are not OLPs, however. First and foremost, double labelling for *Fgfr3* and *Pdgfra* (a marker of early OLPs) demonstrates that these mark separate populations of cells. The *Fgfr3*$^+$ and *Pdgfra*$^+$ cell populations appear at different times and initially their distributions are different. Moreover, the number and distribution of *Fgfr3*$^+$ cells was unaltered in neonatal *Pdgfa*-null spinal cords, which have very few *Pdgfra*$^+$ OLPs and oligodendrocytes (Fruttiger et al., 1999). This argues strongly that the large majority of *Fgfr3*$^+$cells revealed by our in situ hybridisation protocol are not OLPs. Bansal et al. (Bansal et al., 1996) have shown that OLPs do express *Fgfr3* mRNA in culture but only at a low level during the earlier stages of the lineage. Presumably this is below our limit of detection in situ. OLPs upregulate *Fgfr3* strongly just prior to oligodendrocyte differentiation (Bansal et al., 1996) but these presumably represent a small subset of OLPs in the embryonic spinal cord and do not feature in our analysis.

Fgfr3-positive cells co-expressed mRNA encoding glutamine synthetase (Glns; EC 6.3.1.2). In the CNS, Glns is an accepted marker of mature astrocytes (Norenberg and Martinez-Hernandez, 1979; Stanimirovic et al., 1999) but it is also expressed in oligodendrocytes (Domercq et al., 1999) and OLPS (Baas et al., 1998). Glns has not previously been ascribed to neuroepithelial precursors or immature astrocytes in the embryo, although *Glns* transcripts have been detected in the rat brain by northern blot as early as E14. To our knowledge, Glns has not been described in neurones except in pathological situations such as Alzheimer's disease (Robinson, 2000). Therefore, we are confident that the (*Fgfr3*$^+$, *Glns*$^+$) double-positive cells described here are glial cells. Taken together with the evidence against them being OLPs (see above), it seems likely that they correspond to immature and mature astrocytes. This is strongly supported by the observations that *Fgfr3*$^+$ cells co-express Gfap protein and/or mRNA in (1) the formative white matter of the normal developing spinal cord and (2) cultures of dissociated spinal cord cells.

Neuroepithelial origins of astrocytes

Fgfr3 was expressed in two domains of the spinal cord neuroepithelium separated by an *Fgfr3*-negative region. This was true of both rodent and avian embryos though it was more obvious in the latter. The *Fgfr3*-negative region corresponds roughly to the pMN domain of the VZ that generates somatic motoneurones followed by *Pdgfra*$^+$ OLPs (Sun et al., 1998; Rowitch et al., 2002). Therefore, our data indicate that OLPs and astrocytes originate from separate precursors that reside in different parts of the VZ. How does this fit with other ideas about the origin of astrocytes? One hypothesis is that astrocytes arise by transdifferentiation of radial glia, after the latter have fulfilled their role as cellular substrates for radial migration of neuronal progenitors (Bignami and Dahl, 1974; Choi et al., 1983; Benjelloun-Touimi et al., 1985; Voigt, 1989; Culican et al., 1990). This could be compatible with our *Fgfr3* expression data, as radial glia have their cell bodies close to the ventricular

surface. However, radial glia are distributed all around the spinal cord lumen, unlike *Fgfr3*, so one would have to postulate that only a subset of radial glia express *Fgfr3*.

In double-knockout mice that lack the two basic helix-loop-helix (bHLH) transcriptions factors Olig1 and Olig2, the pMN domain of the VZ undergoes a homeotic transformation into p2, its immediate dorsal neighbour (Rowitch et al., 2002). As a result, pMN no longer generates motoneurones followed by OLPs, but instead produces V2 interneurones followed by astrocytes (Zhou and Anderson, 2002; Takebayashi et al., 2002). By implication, this is the usual fate of p2 precursors in wild-type mice. This is consistent with our observation that *Fgfr3*$^+$ astrocytes apparently originate within an extended part of the ventral VZ, including p2 but excluding pMN. Our *Fgfr3* expression data are also consistent with previous fate mapping experiments in chick-quail chimeras, which indicated that astrocytes are generated from dorsal as well as ventral parts of the VZ, whereas OLPs are generated only from ventral territory (Pringle et al., 1998). It remains to be seen whether astrocytes that are generated from distinct neuroepithelial domains (p3 or p2, say) have identical properties or whether they are functionally specialised – for modulating synaptic activity or interacting with blood vessels, for example.

Production of ventral cell types such as motoneurones, interneurones and OLPs is dependent on Shh signalling. As many *Fgfr3*-expressing astrocyte precursors appear to originate in p3, p2 and other ventral domains, we might expect that production of astrocytes might also depend on Shh signalling. However, we found that astrocytes developed in explant cultures of ventral neural tube either in the presence or absence of Shh activity. Our data imply that astrocytes are specified by different mechanisms than OLPs – at least, they demonstrate that astrocyte and OLP production are not obligatorily linked. In fact, there is evidence that more than one signalling pathway can lead to astrocyte development in vitro (Rajan and McKay, 1998). Because astrocytes can be formed from dorsal as well as ventral neuroepithelium, it remains possible that 'ventral' astrocytes might normally be under Shh control, but that by blocking Shh signalling we uncover an alternative 'dorsal' pathway for astrocyte development.

It has been reported that there are glial-restricted precursor cells (GRPs) in the embryonic rat spinal cord that are dedicated to the production of astrocytes and oligodendrocytes (Rao and Mayer-Proschel, 1997; Herrera et al., 2001). This seems to conflict with current evidence that oligodendrocytes and astrocytes are generated from different precursors in the embryonic spinal cord (Lu et al., 2002; Rowitch et al., 2002; Zhou and Anderson, 2002) (this paper). A possible reconciliation might be that GRPs with the potential to generate both astrocytes and oligodendrocytes are formed in all parts of the spinal cord VZ but are constrained in vivo to generate only astrocytes or only oligodendrocytes, depending on the signals in their local environment (i.e. where they are located) (for a review, see Rowitch et al., 2002).

Fgfr3 regulates Gfap expression in grey matter astrocytes

Astrocytes with distinct, heritable morphologies have been described in cultures of rat spinal cord cells (Fok-Seang and Miller, 1992). Astrocytes in different parts of the CNS differ in morphology or function in vivo too, suggesting that they might fulfil different, region-specific functions. In addition, astrocytes in white matter tracts generally have smaller cell bodies with more and longer processes compared to their counterparts in grey matter (Connor and Berkowitz, 1985). For this reason, white matter astrocytes are sometimes referred to as 'fibrous' and those in grey matter as 'protoplasmic' or 'velous'. White matter astrocytes also express high levels of Gfap, whereas grey matter astrocytes contain little or no immunoreactive Gfap.

Fibrous and protoplasmic astrocytes might develop from separate lineages (Connor and Berkowitz, 1985). However, our observation that Gfap is upregulated in grey matter astrocytes of *Fgfr3*-null mice provides strong in vivo evidence that extracellular signals might be required to maintain their normal Gfap-negative phenotype. This is consistent with a report that adding Fgf2 to cultured astrocytes downregulates Gfap mRNA and protein and causes their morphology to change (Reilly et al., 1998). Fgf2 and other known Fgfr3 ligands such as Fgf9 are made by, and presumably released from, many CNS neurones (Eckenstein et al., 1991; Cotman and Gomez-Pinilla, 1991; Woodward et al., 1992; Gomez-Pinilla et al., 1994; Kuzis et al., 1995). One possible reason that white matter astrocytes express high levels of Gfap in wild-type mice might be that they are denied exposure to *Fgfr3* ligands in axon tracts – perhaps because Fgf, like Pdgf, is secreted from neuronal cell bodies but not from axons (Fruttiger et al., 2000).

Upregulation of Gfap in the *Fgfr3*-null mouse is mindful of the astrocyte response to CNS injury or disease – so-called reactive gliosis or astrocytosis (for reviews, see Ridet et al., 1997; Norton, 1999). It would be interesting to know whether interruption of signalling through Fgfr3 is somehow involved in the astrocyte reaction to injury. However, it is unlikely to be straightforward, because Gfap upregulation in the *Fgfr3*-null animals does not occur until around 2 months of age, suggesting that it is an indirect effect. In addition, the data from the *Fgfr3*-null mouse are difficult to square with the observation that intra-ventricular injection of Fgf2 has been reported to increase the number of Gfap$^+$ reactive astrocytes (Unsicker, 1993).

Most grey matter (protoplasmic) astrocytes possess many short sheet-like processes containing little, if any, Gfap (Connor and Berkowitz, 1985). It has been suggested that this morphology might help them to infiltrate the neuropil and surround axonal terminals, synapses and neuronal cell bodies, consistent with one of their proposed roles in neurotransmitter metabolism (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979). It will be interesting to see if the reactive astrocytes in *Fgfr3*-null mice are defective in neurotransmitter metabolism and whether this contributes to the premature death of the animals.

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