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An *Fgfr3-iCreER^{T2}* transgenic mouse line for studies of neural stem cells and astrocytes

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Abstract

The lack of markers for astrocytes, particularly grey matter astrocytes, significantly hinders research into their development and physiological properties. We previously reported that fibroblast growth factor receptor 3 (*Fgfr3*) is expressed by radial precursors in the ventricular zone of the embryonic neural tube and subsequently by differentiated astrocytes in grey and white matter. Here, we describe an *Fgfr3-iCreER^{T2}* PAC transgenic mouse line that allows efficient tamoxifen-induced Cre recombination in *Fgfr3*-expressing cells, including radial glial cells in the embryonic neural tube and both fibrous and protoplasmic astrocytes in the mature central nervous system. This mouse strain will therefore be useful for studies of normal astrocyte biology as well as their responses to CNS injury or disease. In addition, *Fgfr3-iCreER^{T2}* drives Cre recombination in all neurosphere-forming stem cells in the adult spinal cord and at least 90% of those in the adult forebrain subventricular zone (SVZ). We made use of this to show that there is continuous accumulation of all major interneuron sub-types in the olfactory bulb (OB) from postnatal day 50 (P50) until at least P230 (~8 months of age). It therefore seems likely that adult-born interneurons integrate into existing circuitry and perform long-term functions in the adult OB.

Keywords

Fgfr3; Cre-lox; transgenic mice; astrocytes; radial glia; neural stem cells; spinal cord; SVZ; olfactory bulb; interneurons

Introduction

Astrocytes are the most abundant cells in the adult mouse brain, yet the paucity of molecular markers with which to identify astrocytes in situ means that they remain relatively poorly characterized. Two broad classes of astrocyte are recognized – fibrous astrocytes, found mainly in white matter and protoplasmic astrocytes in the grey matter. Within these broad

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Author Contributions

TM, MG and NK made the *Fgfr3-iCreER^{T2}* mice. KMY characterized the mice, performed the experiments, prepared the figures and wrote the paper with WDR. WDR, NK and KMY conceived the experiments and WDR obtained funding.

groupings there might be functionally specialized subtypes, for region-specific gene expression differences have been reported (Hochstim et al., 2008). At least some astrocytes develop by direct trans-differentiation of radial glial cells after they have undergone their final divisions (Hirano and Goldman, 1988; Voigt, 1989). Based on the development of their densely ramified fine processes and exclusive territories (Nagy and Rash, 2003; Bushong et al., 2004), they are said to reach maturity between postnatal day 17 (P17) and P30. Unlike the neuronal or oligodendroglial lineages, we have no simple way of distinguishing astrocytes at different stages of maturation, which impedes study of their differentiation and plasticity in the normal or injured brain.

Glial fibrillary acidic protein (GFAP) (Bignami and Dahl, 1974) is commonly used as an astrocyte marker but, while GFAP is expressed strongly by fibrous astrocytes in white matter and at the pial surface, it is absent from the vast majority of grey matter (protoplasmic) astrocytes in the normal healthy CNS. S100 β is another glial marker (Ludwin et al., 1976) that is used to mark GFAP-negative protoplasmic astrocytes. However, this protein is also expressed by some oligodendrocyte lineage cells (Vives et al., 2003; Zuo et al., 2004; Hachem et al., 2005). Therefore, identifying astrocytes unambiguously in vivo has proven difficult. We previously reported that the *fibroblast growth factor receptor 3* (*Fgfr3*) transcripts are expressed in the ependymal zone (EZ) of the embryonic spinal cord and subsequently become restricted to astrocytes in the grey and white matter of the postnatal cord (Pringle et al., 2003). *Fgfr3* has been used subsequently for situ studies of astrocyte development (Agius et al., 2004; Hochstim et al., 2008).

Fgfr3 is also expressed in the ventricular zone (VZ) of the developing brain (Bansal et al., 2003). A constitutive activating mutation of FGFR3 has also been associated with increased MAPK activity and increased proliferation of cells in the VZ (Inglis-Broadgate et al., 2005; Thomson et al., 2007). These data indicate that FGFR3 is expressed by neuroepithelial precursors and/or radial glial cells in the brain, as in the spinal cord. Many *Fgfr3*⁺ cells in the postnatal brain are found outside the VZ (Bansal et al., 2003), similar to the spinal cord (Pringle et al., 2003). *Fgfr3* expression both in vivo and in cultured neural cells has been linked to oligodendrocyte (OL) lineage cells as well as astrocytes (Miyake et al., 1996; Bansal et al., 2003; Oh et al., 2003). However, *Fgfr3* is expressed at a low level in early oligodendrocyte precursors (OLPs) being up-regulated only transiently as they start to differentiate into oligodendrocytes (Bansal et al., 1996), so it is to be expected that the great majority of *Fgfr3*⁺ cells in the postnatal CNS should be astrocytes, not OL lineage cells. Consistent with this, Cahoy et al. (2008) immuno-purified astrocytes from early postnatal brains (P1-P30) of *S100 β -eGFP* mice and showed that *Fgfr3* mRNA was highly enriched relative to other purified neural cell types on Affymetrix gene microarrays.

Here, we describe the generation and characterization of a phage artificial chromosome (PAC) transgenic mouse line that expresses tamoxifen-inducible Cre (“codon-improved” version, iCreERT²) under *Fgfr3* transcriptional control. This has allowed us to identify and study *Fgfr3*⁺ cells in the developing and/or adult CNS. We have confirmed that *Fgfr3* is expressed by radial glial stem cells in the embryonic brain and spinal cord and by fibrous and protoplasmic astrocytes in the postnatal CNS. In addition, we show that *Fgfr3* is expressed by adult neural stem cells located within the SVZ of the forebrain and the EZ of

the spinal cord. We followed the development of olfactory bulb (OB) interneurons from SVZ stem cells during adulthood and showed that all identifiable interneuron subtypes accumulate and survive long-term (> 6 months) in the granule and periglomerular layers of the OB. We expect that our *Fgfr3-iCreER^{T2}* line will be useful for further studies of OB neurogenesis as well as studies of astrocytes in the postnatal CNS. In addition, it is likely that the line will find uses for studies of non-CNS tissues that normally express *Fgfr3*, such as developing cartilage and bone.

Materials and Methods

Transgenesis

The mouse genomic PAC library RPCI 21 from the UK Human Genome Mapping Project Resource Centre was screened with a 900 bp PCR-generated fragment from a rat *Fgfr3* cDNA, corresponding to most of the extracellular domain. One positive clone (608P12) was selected for modification. It contained an ~180 kb insert, including 37 kb upstream and 130 kb downstream of the *Fgfr3* gene. The targeting construct was designed to insert an *iCreER^{T2}-SV40polyA* cassette into the first coding exon (exon 2) of the *Fgfr3* gene, fusing it to the endogenous initiation codon and deleting 58 bp immediately downstream of it. *iCreER^{T2}* (Claxton et al., 2008) is a fusion between *iCre* (excluding the nuclear localization signal) (Shimshek et al., 2002) and the ERT2 component of CreER^{T2} (Indra et al., 1999). Recombination and selection of correctly-recombined clones and removal of fragments from the pPAC4 vector backbone were carried out as described previously (Lee et al., 2001; Rivers et al., 2008). The modified PAC was digested with SgfI, which recognizes a single site in the PAC vector backbone. The linear PAC was purified by pulsed field gel electrophoresis and transgenic mice were generated by pronuclear injection. Five founders were identified by Southern blotting. Three of these produced fertile transgenic offspring, which gave indistinguishable *Cre* expression patterns by in situ hybridization. All data described here refer to founder #4-2. These and other Cre transgenics can be requested online at <http://www.ucl.ac.uk/~ucbzwdr/Richardson.htm> ("request a mouse strain").

Genotyping and embryo staging

Genotyping was performed by PCR using primers iCre250 (GAG GGA CTA CCT CCT GTA CC) and iCre880 (TGC CCA GAG TCA TCC TTG GC) which amplify a 630 bp fragment. The amplification programme was: 94°C/ 4 minutes, followed by 33 cycles of 94°C/ 30s, 61°C/ 45s, 72°C/ 1 minute and a final step of 72°C/ 10 minutes. Heterozygous *Fgfr3-iCreER^{T2}* mice were crossed to *R26R-GFP* (Mao et al., 2001), *R26R-YFP* (Srinivas et al., 2001) or *Z/EG* (Novak et al., 2000) Cre-conditional reporters, and double-heterozygous offspring were selected for analysis. *R26R-GFP* and *R26R-YFP* transgenes were identified by PCR of tail DNA as described (Psachoulia et al., 2009). The *Z/EG* transgene was identified by whole mount β -Galactosidase staining of tail tissue.

For timed matings, males and females were caged together overnight and vaginal plugs scored the following morning. Midday of the day of the vaginal plug was designated embryonic day 0.5 (E0.5). Pregnant females were killed by CO₂ inhalation and the embryos removed. Embryonic ages were confirmed by morphological criteria (Theiler, 1972).

Tamoxifen administration

Tamoxifen was dissolved in corn oil at a concentration of 40 mg/ml and administered by oral gavage. Adult mice (P50 or older) received 200 mg/Kg body weight once a day for five consecutive days. Pregnant females received a single dose of 200 mg/Kg body weight. Time after tamoxifen administration is denoted as e.g. P50+7, where +7 refers to the number of days after the *first* dose given on P50.

BrdU administration

BrdU (Sigma) was dissolved in phosphate buffered saline (PBS) at 20 mg/ml and 50 µl was administered intra-peritoneally to adult mice four times in a 24 hour period (6am, noon, 6pm and midnight).

Tissue preparation and sectioning

Adult mice were perfusion-fixed with 4% (w/v) paraformaldehyde (PFA) in PBS. Brain and spinal cord tissues from adults or embryos were immersion fixed in 4% PFA in PBS overnight at 4°C, cryo-protected overnight at 4°C in 20% (w/v) sucrose in water [pre-treated with diethylpyrocarbonate (DEPC)], embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Raymond Lamb Ltd.) and frozen on dry ice. Tissue was stored at -80°C until needed. Cryosections were cut for immunohistochemistry (30 µm) or in situ hybridization (18-20 µm). Adult brain sections for in situ hybridization were collected on the surface of DEPC-treated PBS, transferred onto glass slides and allowed to dry in air at 20-25°C. Adult brain and spinal cord sections were immuno-labelled as floating sections and transferred to glass slides for mounting and microscopy. Embryonic brain and spinal cord sections were collected directly onto coated glass slides.

Immunohistochemistry

Immunohistochemistry was performed using methods and antibodies previously described (Young et al., 2007; Rivers et al., 2008). Additional antibodies were: rabbit anti-Aquaporin-4 (Chemicon; 1:500); guinea-pig anti-GLAST (Chemicon; 1:5000); mouse anti-RC2 monoclonal IgM supernatant (Developmental Studies Hybridoma Bank; 1:4).

In situ hybridization

The *Fgfr3* RNA hybridization probe was prepared as previously described (Pringle et al., 2003). For the *iCreER^{T2}* probe, *iCreER^{T2}* coding sequences were PCR amplified using primers that added a 5' SalI site and a 3' EcoRI site. The PCR product was cloned into pBluescript along with a 3' *SV40-polyA* sequence. The antisense probe was transcribed with T3 RNA polymerase (Promega) from SalI-linearized template in the presence of digoxigenin- or fluorescein-conjugated nucleotide labelling mix (Roche). For details see <http://www.ucl.ac.uk/~ucbzwdr/Richardson.htm> ("protocols").

Neurosphere Cultures

Primary neurosphere cultures were generated from the entire spinal cord or the micro-dissected SVZ of *Fgf3-iCreER^{T2} : R26R-YFP* mice at various times after tamoxifen administration at P70 (i.e. P70+7, P70+14, P70+21 and P70+56). Separate cultures were

established from three individual mice. Tissue was chopped into small pieces, digested with Trypsin-EDTA (Invitrogen) at 37°C for 12 minutes and digestion was stopped with soybean trypsin inhibitor (Sigma). Cells were triturated in calcium- and magnesium-free Earles Buffered Salt Solution (Invitrogen) to generate a single cell suspension. Dissociated cells from each single mouse were plated in one (SVZ cells) or two (spinal cord cells) six-well tissue culture plates in 90% Neurocult basal medium for neural stem cells (mouse) /10% Neurocult neural stem cell proliferation supplement (mouse) (Stem Cell Technologies), plus 10 ng/ml basic fibroblast growth factor (Roche), 20 ng/ml epidermal growth factor (Sigma) and 4 µg/ml heparin sodium salt (Sigma). Cultures were maintained at 37°C in a 5% CO₂ atmosphere. Half of the medium of spinal cord cultures was replaced at 7 days in vitro. The proportion of YFP⁺ neurospheres in each culture was determined using an inverted fluorescent microscope at either 7 days (SVZ) or 14 days (spinal cord) in vitro.

Microscopy and data analysis

Brain and spinal cord sections were imaged using a Zeiss Axioplan fluorescence microscope with a Hamamatsu camera controlled by Simple PCI software. Images were collected as single optical scans using an Ultraview confocal microscope (Perkin Elmer). For quantitative analysis a series of non-overlapping images (20X objective lens) were collected from selected adult brain regions (at least 8 micrographs per region per brain section). Three brain sections from each of three mice were counted for each staining condition (at least 300 labelled cells in total). The brain regions analyzed are illustrated in supplementary Fig. S1. Statistical comparisons were made by a t-test or, when there were multiple regions or time points, by ANOVA. Differences were considered to be statistically significant at $P < 0.05$. Co-localization of antigens was confirmed by generating XZ and YZ views of micrographs using Volocity software (Perkin Elmer).

Results

Fgfr3 as a marker for cortical astrocytes

Following from our previous study of mouse spinal cord (Pringle et al., 2003), we examined *Fgfr3* as a candidate marker for astrocytes in the postnatal brain. By in situ hybridization we detected many *Fgfr3*⁺ cells in the adult mouse cerebral cortex (Fig. 1). These cells did not co-express NG2, OLIG2 or NeuN immunoreactivity (Fig. 1a-c), indicating that they were not oligodendrocyte lineage cells or neurons. The majority of *Fgfr3*⁺ cells were also negative for GFAP, but all GFAP⁺ cells co-expressed *Fgfr3* (Fig. 1d). Since most cortical astrocytes do not express GFAP, this labelling pattern was consistent with the notion that *Fgfr3* is specifically expressed by astrocytes in the adult mouse cortex.

Expression of an *Fgfr3-iCreER*^{T2} PAC transgene in the CNS

To characterize the *Fgfr3*⁺ cells further, we generated transgenic mouse lines that express iCreER^{T2} under the transcriptional control of *Fgfr3* in a PAC (Fig. 1e and Methods). We obtained five founders, three of which transmitted the *Fgfr3-iCreER*^{T2} transgene to their offspring. We initially characterized each founder by in situ hybridization for *Fgfr3* and *iCreER*^{T2} on adjacent sections of embryonic spinal cords. *Fgfr3* and *iCreER*^{T2} displayed very similar expression patterns at all ages examined (supplementary Fig. S2). At the earliest

times examined (embryonic day 11.5, E11.5), expression was restricted to cell bodies in the ependymal zone (EZ). We identified these EZ cells as pluripotent radial precursors that generate neurons, astrocytes and oligodendrocytes during embryonic development (supplementary Fig. S2). Subsequently, *Fgfr3*- and *iCreER^{T2}*-positive cells appeared to migrate away from the EZ into the developing grey and white matter, where they persisted long-term (supplementary Fig. S2).

In the postnatal spinal cord and brain, many *Fgfr3*⁺ cells were detected in grey and white matter (Fig. 1 and supplementary Fig. S2). In adult *Fgfr3-iCreER^{T2}* mice, the *Fgfr3*⁺ cells in both grey and white matter co-expressed *iCreER^{T2}* (Fig. 1f). We tentatively concluded that expression of the *Fgfr3-iCreER^{T2}* transgene mimics normal *Fgfr3* expression in the embryonic and adult spinal cord and adult forebrain, so we characterized the transgene further by crossing it into a Cre-conditional reporter background.

***Fgfr3-iCreER^{T2}* marks protoplasmic and fibrous astrocytes**

Fgfr3-iCreER^{T2} was crossed into the *Rosa26R-eYFP (R26R-YFP)* background (Srinivas et al., 2001), tamoxifen was administered on five consecutive days starting on ~P50 and the mice were analyzed at various times post-tamoxifen by immunolabelling for YFP. One day after the final dose of tamoxifen (five days after the initial dose, P50+5), no YFP-labelled cells were detected anywhere in the CNS, either because recombination had not yet occurred or because the level of YFP protein had not yet accumulated to detectable levels. However, at P50+7 many YFP⁺ cells were found throughout the grey matter of the brain (Fig. 2) and spinal cord (supplementary Fig. S2). These cells were close-packed and filled almost the entire grey matter volume with YFP fluorescence. Within this sea of fluorescence were scattered “holes”, corresponding to cells that had escaped labelling (e.g. Fig. 2a-c, arrowheads). This pattern of labelling was consistent with the idea that the YFP-labelled cells were astrocytes, and indicated that the efficiency of *R26R-YFP* reporter gene activation was high, though less than 100%. We never observed any YFP⁺ cells in *Fgfr3-iCreER^{T2}; R26R-YFP* mice that had not received tamoxifen.

Immuno-labelling for the glial marker S100 β supported the idea that YFP⁺ cells in the cortex were astrocytes. The great majority of YFP⁺ cells in *R26R-YFP* reporters were S100 β ⁺ ($94 \pm 4\%$ of YFP⁺ cells) and most of these were GFAP-negative (Fig. 3a-c, m). This fits the idea that the YFP⁺ cells are protoplasmic astrocytes, which normally express low or undetectable levels of GFAP. The minority of cortical YFP⁺ cells that was GFAP⁺ ($18 \pm 4\%$) tended to be associated with blood vessels or the pial surface (Fig. 3d). Those that contacted blood vessels frequently also expressed Aquaporin-4 (Fig. 3e). We found that a very small number of YFP⁺ cortical cells co-expressed the neuronal marker NeuN ($0.2 \pm 0.4\%$ of YFP⁺ cells) or the oligodendrocyte lineage marker OLIG2 ($1.0 \pm 0.6\%$ of YFP⁺ cells) (Fig. 3f-i, m). These double-labelled cells did not localize to any particular region of the forebrain. Many (around half) of the OLIG2⁺, YFP⁺ cells expressed a low level of OLIG2 relative to the OLIG2⁺, YFP-negative cells (Fig. 3g). We do not know the significance of this. Although S100 β is widely used as a marker of protoplasmic astrocytes it is also known to be expressed by some oligodendrocyte lineage cells (Hachem et al., 2005; Vives et al., 2003). To distinguish oligodendrocytes from astrocytes we performed triple immunolabelling for YFP,

S100 β and OLIG2. The great majority ($92 \pm 5\%$) of YFP $^{+}$ cells co-expressed S100 β but not OLIG2 (Fig. 3r), confirming them as astrocytes.

In coronal sections of P50+7 corpus callosum, a major white matter tract, the vast majority ($95 \pm 3\%$) of YFP $^{+}$ cells were GFAP $^{+}$ (Fig. 3k-m, o), identifying them as fibrous astrocytes. This is possibly an underestimate, as GFAP is filamentous and not always expressed in the cell body. A very small proportion ($1.0 \pm 0.8\%$) of YFP $^{+}$ cells co-labelled for OLIG2 (Fig. 3j, m) and none co-labelled for NeuN (Fig. 3m). A tiny minority of all SOX10 $^{+}$ oligodendrocyte lineage cells ($0.07\% \pm 0.01\%$) co-expressed YFP $^{+}$. Similarly, a tiny fraction of NG2 $^{+}$ oligodendrocyte precursors ($0.3\% \pm 0.1\%$) were YFP $^{+}$. Taken together, these data demonstrate that recombination in the *R26R-YFP* reporter background is both efficient and astrocyte-specific.

To examine the detailed morphology of labelled cells we used the *Z/EG* reporter, in which eGFP is expressed in a Cre-inducible manner from a synthetic promoter composed of *CMV* and *β -actin* promoter elements (Novak et al., 2000). *Z/EG* reporters recombined less efficiently (fewer cells labelled) than *R26R-YFP* (compare images Fig. 2b and Fig. 2f). In P50+7 corpus callosum only $12 \pm 1\%$ of GFAP $^{+}$ astrocytes were GFP $^{+}$ in the *Z/EG* reporter (Fig. 3n, p), whereas $89 \pm 4\%$ were YFP $^{+}$ in *R26R-YFP* reporters. In the cortex, $14 \pm 4\%$ of S100 β $^{+}$, OLIG2-negative protoplasmic astrocytes were GFP $^{+}$ in *Z/EG* reporters, compared to $95 \pm 1\%$ in *R26R-YFP* (Fig. 3q-s). Nevertheless, expression of GFP in individual, isolated cells was very high in *Z/EG* reporters so that even fine processes were visible by GFP immuno-labelling. The morphologies of GFP $^{+}$ cells in the corpus callosum (Fig. 2g) and at the pial surface (Fig. 2h) resembled fibrous white matter astrocytes and sub-pial astrocytes, respectively. In the cortical grey matter, GFP $^{+}$ cells had a dense halo of very fine processes around their cell bodies, giving them a distinct “bushy” or “fuzzy” morphology typical of protoplasmic astrocytes (Fig. 2i).

Long term stability of *Fgfr3*-expressing astrocytes in the cortex

To investigate the long-term fates of *Fgfr3*-expressing cells in the adult CNS, we immunolabelled coronal forebrain sections of P50+14 and/or P50+80 *Fgfr3-iCreER^{T2}; R26R-YFP* mice for YFP and either GFAP (to identify fibrous astrocytes) or with S100 β and OLIG2 (to identify S100 β $^{+}$, OLIG2-negative protoplasmic astrocytes) (Fig. 4).

In the cortex between P50+7 and P50+80 there was no significant change in the proportion of YFP $^{+}$ protoplasmic astrocytes ($92 \pm 5\%$ versus $97 \pm 1\%$, respectively), nor was there a change in the proportion of YFP $^{+}$ cells that labelled for OLIG2 ($1.0 \pm 0.6\%$ versus $1.0 \pm 1.0\%$) or NeuN ($0.2 \pm 0.4\%$ versus $0.3 \pm 0.3\%$).

In the corpus callosum, there was no significant change in the proportion of YFP $^{+}$ cells that were GFAP $^{+}$ fibrous astrocytes ($> 95\%$ at all ages from P50+7 to P50+80) (Fig. 4a, b, e). However, we found a small but significant accumulation of YFP $^{+}$ oligodendroglial cells with time (Fig. 4c-e). Between P50+14 and P50+80 the proportion of YFP $^{+}$ cells that was NG2 $^{+}$ increased ~3-fold from ~0.2% to ~0.6%, and the proportion of YFP $^{+}$ cells that was SOX10 $^{+}$ increased from ~0.8% to ~2.3% (significance, $P < 0.01$; Fig. 4e). These presumptive oligodendrocyte lineage cells might be derived from *Fgfr3* $^{+}$ astrocytes. However, a small

number of oligodendrocytes in the adult corpus callosum is thought to be generated throughout life by SVZ stem cells (Menn et al., 2006; Rivers et al., 2008). Therefore it seemed possible that in addition to labelling astrocytes our *Fgfr3-iCreERT2* transgene might target SVZ stem cells, which share some of their properties with astrocytes. We confirmed this supposition (below).

***Fgfr3*⁺ neural stem cells in the spinal cord ependymal zone and forebrain SVZ**

We previously noted that the spinal cord EZ was YFP-labelled in embryonic *Fgfr3-iCreERT2* : *R26R-YFP* mice. This EZ labelling also persisted in the adult spinal cord (supplementary Fig. S2). In addition, the forebrain SVZ was heavily YFP-labelled (Fig. 2e, arrows). This suggested that *Fgfr3-iCreERT2* might induce recombination in neural stem cells, which are present in both the EZ and the SVZ (Weiss et al., 1996; Doetsch et al., 1999; Hamilton et al., 2009). Consistent with this, some YFP⁺ cells in the SVZ co-expressed GFAP, which in addition to identifying fibrous astrocytes is also a marker of SVZ stem cells (“subependymal astrocytes” or “type-B cells”) (Doetsch et al., 1999; Laywell et al., 2000) (Fig. 5a).

To test for stem cell labelling we generated neurosphere cultures from the SVZ and spinal cords of *Fgfr3-iCreERT2* : *R26R-YFP* mice at increasing times (7 to 80 days) post-tamoxifen (Fig. 5b-c) and determined the proportion of neurospheres that was YFP⁺ in each culture (Fig. 5d). At all times post-tamoxifen, 100% of spinal cord-derived neurospheres were uniformly YFP⁺ (Fig. 5d). EZ stem cells are the only cells capable of generating neurospheres in the normal healthy spinal cord, so all adult EZ stem cells must be targeted by the *Fgfr3-iCreERT2* transgene. In SVZ cultures, both stem cells (type-B) and progenitor cells (type-C) have the capacity to generate neurospheres (Young et al., 2007). If only the stem cell population expresses *Fgfr3-iCreERT2*, then only a fraction of neurospheres developing in SVZ cultures should be YFP⁺. However, this fraction would be expected to increase, the longer the delay between tamoxifen administration in vivo and establishment of the SVZ cell cultures. This is because YFP⁺ stem cells give rise to YFP⁺ intermediate progenitor cells, while pre-existing (YFP-negative) progenitors generate migratory neuroblasts that leave the SVZ to join the rostral migratory stream (RMS) and move towards the olfactory bulb (OB). This is what we found experimentally. Only $31 \pm 11\%$ of neurospheres were YFP⁺ in SVZ-derived cultures that were established at the shortest time post-tamoxifen (P70+7) (Fig. 5d). However, the proportion of neurospheres that was YFP⁺ increased with time post-tamoxifen, to $89 \pm 4\%$ at P70+56 and $90 \pm 6\%$ at P70+80 (Fig. 5d). These data indicate that ~90% of SVZ stem cells recombine following tamoxifen administration (i.e. recombination efficiency $\cong 90\%$), similar to the recombination rate in fibrous and protoplasmic astrocytes. Consistent with this, BrdU labelling experiments in vivo showed that $91 \pm 2\%$ of BrdU⁺ cells in the SVZ of adult P50+80 *Fgfr3-iCreERT2* : *R26R-YFP* mice were YFP⁺ (four BrdU injections in 24 hours; Fig. 5e).

If *Fgfr3-iCreERT2* is expressed in migratory neuroblasts (type-A cells), one would expect to find YFP-labelling of PSA-NCAM⁺ neuroblasts in the SVZ, RMS and OB at short times post tamoxifen. Immuno-labelling coronal sections of P50+7 brains showed that a decreasing proportion of PSA-NCAM⁺ neuroblasts was YFP⁺ as one moved rostrally from

the SVZ to the OB ($27 \pm 9\%$ versus $0.4 \pm 0.4\%$, respectively) (Fig. 6a-c, g). The gradient of recombination (YFP labelling) from SVZ to OB suggests that the *Fgfr3-iCreER^{T2}* transgene is not transcribed in PSA-NCAM⁺ neuroblasts directly, but that neuroblasts inherit an active YFP reporter from SVZ stem cells, via intermediate progenitors (neither stem cells nor intermediate progenitors express PSA-NCAM). If this is correct, then an increasing proportion of neuroblasts should become YFP-labelled with increasing time post-tamoxifen.

To test this, coronal sections of SVZ, RMS and OB from P50+14, P50+80 and P50+180 *Fgfr3-iCreER^{T2} : R26R-YFP* mice were immuno-labelled for PSA-NCAM and YFP (Fig. 6d-g). At P50+14 there was still a rostro-caudal gradient in the proportion of neuroblasts labelled. However by P50+80 the proportion of PSA-NCAM⁺ cells that was YFP⁺ had reached ~90% in the SVZ, RMS and OB and this did not increase further, even at P50+180 (Fig. 6d-f, g). These data indicate that the *Fgfr3-iCreER^{T2}* transgene is not expressed by migrating neuroblasts but that, with time, they inherit the recombined *YFP* allele from *Fgfr3⁺* stem cells in the SVZ. Furthermore, as progenitor cells have a limited capacity to generate neuroblasts and must be continually replenished from SVZ stem cells, the continued long-term production of YFP⁺ neuroblasts (for ~6 months) confirms that the true SVZ stem cell population was labelled in *Fgfr3-iCreER^{T2} : R26R-YFP* mice.

SVZ-derived interneurons accumulate in the olfactory bulb and survive long-term

The evidence above demonstrates that *Fgfr3* is expressed by SVZ stem cells (type-B), but not by SVZ progenitors (type-C) or migratory neuroblasts (type-A). Furthermore we found that no NeuN⁺ cells in the OB co-express YFP in P50+7 *Fgfr3-iCreER^{T2} : R26R-YFP* mice, demonstrating that post-mitotic OB neurons are themselves *Fgfr3*-negative (Fig. 6h). We took advantage of this to investigate the rate of arrival and accumulation of SVZ- derived interneurons in the adult OB. We administered tamoxifen to *Fgfr3-iCreER^{T2} : R26R-YFP* mice on P50 and analyzed OB sections by immuno-labelling for YFP and NeuN on P50+7, P50+14, P50+80 and P50+180 (Fig. 6h-j). In addition we identified interneuron subtypes by immunolabelling for Calretinin, Calbindin or Tyrosine Hydroxylase (Fig. 6k-m). The proportion of each interneuron subtype that was YFP-labelled increased between P50+14 and P50+80 and again between P50+80 and P50+180 (Fig. 6n), as expected if new adult-born neurons arrive in the OB from the RMS and survive in the OB for an extended period of time – at least six months. This suggests that the adult-born interneurons integrate into the OB circuitry and fulfil a long-term function in both the periglomerular and granule cell layers.

Discussion

Finding markers that identify both white- and grey matter astrocytes but not other kinds of neural cell in the CNS has been a thorny problem. We showed that *Fgfr3* is expressed by astrocytes in the developing and mature CNS (Pringle et al., 2003) and now we have made an *Fgfr3-iCreER^{T2}* PAC transgenic mouse line that can be used with conditional reporters to label astrocytes in the mature CNS. The efficiency of Cre recombination following tamoxifen administration by oral gavage was very high with the *R26R-YFP* reporter; ~90% of all protoplasmic and fibrous astrocytes could be labelled in the adult mouse brain. This is

sufficient to allow purification of astrocytes (or other *Fgfr3*⁺ cells) by FAC sorting for biochemical studies and could also be useful for conditional gene deletion or over-expression. On the *Z/EG* reporter background recombination was much less efficient, labelling only ~12% of protoplasmic or fibrous astrocytes. Nevertheless, each individual cell was very brightly labelled in the *Z/EG* reporter background, revealing fine detail of the cellular morphology. For some types of experiment (e.g. electrophysiology) this could be a definite advantage.

In general, recombination efficiency depends on both the Cre driver and the Cre-conditional reporter. We have observed with *Fgfr3-iCreER*^{T2} and many other Cre lines, both inducible and constitutive, that *R26R-YFP* (Srinivas et al., 2001) consistently gives the highest recombination rates. This presumably reflects structural features of the reporter transgene such as distance between *lox* sites (shorter distances favouring recombination). Strikingly, we observed practically no recombination in adult *R26R-GFP* reporters (Mao et al., 2001), either with *Fgfr3-CreER*^{T2} or with other inducible Cre lines such as *Pdgfra-CreER*^{T2} (Rivers et al., 2008), although the *R26R-GFP* reporter has many times been used successfully with constitutive Cre lines by ourselves and others. We ascribe the relatively inefficient recombination of *R26R-GFP* to the fact that it contains three *lox* sites, not two as in *R26R-YFP*. On top of this, CreER^{T2} is much less active than constitutive Cre, either because of its altered structure or because it is only transiently activated by tamoxifen, or both. The recombination efficiency of the other reporters we have used, *Z/EG* (Novak et al., 2000) and *R26R-LacZ* (Soriano, 1999), are intermediate between *R26R-YFP* and *R26R-GFP*, with *R26R-LacZ* being rather more efficient than *Z/EG*, although we have not quantified this.

Many markers of mature astrocytes appear to be shared with other glial cell types, particularly radial glia. For example, transgenic lines that express constitutively active Cre under the transcriptional control of human *GFAP* (*hGFAP*) or *brain lipid binding protein* (*BLBP*) activate recombination in radial glia during embryogenesis and hence label all of their progeny, including neurons and oligodendrocytes as well as astrocytes (Malatesta et al., 2003; Anthony et al., 2004; Casper and McCarthy, 2006; Hegedus et al., 2007). An *S100β-eGFP* line (Vives et al., 2003) labels embryonic radial glia and both astrocytes and oligodendrocytes in the postnatal CNS, mimicking endogenous S100β expression. Tamoxifen-inducible CreER^{T2} lines that are available to label astrocytes include those driven by the human GFAP promoter (*hGFAP*) (Ganat et al., 2006; Hirrlinger et al., 2006; Chow et al., 2008) *GLAST* (Mori et al., 2006; Slezak et al., 2007) or *Connexin 30* (*Cx30*) promoters (Slezak et al., 2007). With the exception of *Cx30-CreER*^{T2}, these lines, like our *Fgfr3-iCreER*^{T2}, label radial glia and SVZ stem cells as well as astrocytes. This points to a close relatedness between radial glia and astrocytes, presumably connected to the fact that at least some astrocytes develop by direct trans-differentiation from radial glia (i.e. without an intervening cell division) (Hirano and Goldman, 1988; Voigt, 1989).

We also showed that *Fgfr3* is expressed by stem cells in the postnatal SVZ and RMS but not by the progenitor cells or neuroblasts. The latter is consistent with a report that, following a single pulse of BrdU, most BrdU⁺ cells in the SVZ (mainly progenitor cells) do not express *Fgfr3* mRNA (Frinchi et al., 2008). It is difficult to label the main population of “parenchymal” astrocytes without also labelling “subependymal astrocytes” in the SVZ (the

stem cells), as they share several properties, not only with each other but also with embryonic radial glia. Recent gene array studies of parenchymal astrocytes have identified many new genes that are preferentially expressed by astrocytes in vivo (Cahoy et al., 2008; Lichter-Konecki et al., 2008; Obayashi et al., 2009); these gene sets promise to provide a rich source of new astrocyte-specific markers for the future.

It is unclear whether there are intermediate astrocyte progenitors residing in the parenchyma of the adult brain, analogous to “NG2 cells” or “polydendrocytes” which act as life-long progenitors for oligodendrocytes (Dimou et al., 2008; Rivers et al., 2008; Nishiyama et al., 2009). Approximately 5% of cortical astrocytes have been reported to “regain” stem cell status and to become capable of generating neurospheres in culture following cortical injury (Buffo et al., 2008). This implies that 5% of astrocytes might be more plastic than the rest, suggesting that they might correspond to astrocyte progenitors. We observed low-level OLIG2 expression in a small proportion of cells in *Fgfr3-iCreER^{T2} : R26R-YFP* mice. Perhaps these OLIG2⁺ cells include astrocyte progenitors, because OLIG2 is reportedly expressed by immature, developing astrocytes (Marshall et al., 2005), is up-regulated in astrocytes following injury (Buffo et al., 2005; Cassiani-Ingoni et al., 2006; Magnus et al., 2007) and is required for their proliferation (Chen et al., 2008). However, OLIG2 can be up-regulated in ~80% of astrocytes following a cortical injury (Chen et al., 2008), compared to the ~5% that develop neurosphere-forming capabilities (Buffo et al., 2008).

We have not examined the fates of *Fgfr3*⁺ astrocytes following mechanical injury to the CNS. However, in collaborative experiments to be reported elsewhere (WDR and RMJ Franklin, University of Cambridge, UK; manuscript in preparation) we found that, following experimental focal demyelination in *Fgfr3-CreER^{T2} : R26R-YFP* spinal cords, YFP⁺ reactive astrocytes but no other cell types were generated around the remyelinating lesions. In parallel experiments with *Pdgfra-CreER^{T2} : R26R-YFP* mice, we found few if any YFP-labelled reactive astrocytes. These data suggest that reactive astrocytes are formed from pre-existing parenchymal astrocytes and/or EZ stem cells but not from *Pdgfra*-expressing oligodendrocyte precursors/NG2 cells.

We used the fact that our *Fgfr3-iCre^{T2}* transgene is expressed in SVZ stem cells to assess their contribution to adult neurogenesis in the olfactory bulb (OB). There is a variety of interneuron subtypes in the OB. These include the three major populations of GABAergic interneurons in the periglomerular layer, which can be distinguished by immuno-labelling for the calcium binding proteins Calbindin and Calretinin and the dopamine synthesizing enzyme Tyrosine Hydroxylase. The lifespans and functions of these new interneurons has been a matter of great interest in recent years. We report that newly born neurons (YFP⁺, NeuN⁺) accumulate in the periglomerular and granule neuron layers of the OB for at least 6 months after tamoxifen-induced labelling of the SVZ stem cells. By this time the adult-born interneurons comprise ~15% of all periglomerular interneurons and ~35% of all granule neurons. These data are consistent with a recent study (Imayoshi et al., 2008) in which olfactory bulb neurogenesis was followed using *Nestin-CreER^{T2}* on the *R26R-LacZ* reporter background to label SVZ stem and progenitor cells. These authors demonstrated that adult-born interneurons comprised ~40% of all granule neurons by 6 months post- tamoxifen (Imayoshi et al., 2008). Moreover, it has been established that, in rats, granule neurons that

are born at 2 months of age (identified by BrdU pulse-labelling) are still present at 19 months of age (Winner et al., 2002). Our study complements those results by demonstrating that accumulation and long-term survival of adult-born olfactory interneurons is not limited to granule neurons but extends to three distinct sub-classes of periglomerular interneurons.

supplementary Information

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *Fgfr3* mRNA in astrocytes of the adult mouse cerebral cortex

In situ hybridization for *Fgfr3* (red) was combined with immuno-labelling for a variety of neural cell type-specific markers (green) in coronal forebrain sections of P60 wild type mice. Cells expressing *Fgfr3* transcripts were distributed throughout all regions of the forebrain. Single confocal scans (1 μ m) from the medial cortex demonstrate that *Fgfr3*⁺ cells did not co-express NG2 (a), OLIG1/2 (b) or NeuN (c) immunoreactivity. GFAP-positive cells co-expressed *Fgfr3* (d). (e) A PAC containing ~37 kb upstream and 110 kb downstream of the mouse *Fgfr3* locus was modified by insertion of *iCreERT2* into exon 2 immediately

downstream of the endogenous initiation codon. (f) Double fluorescence in situ hybridization confirmed that *Fgfr3* (R3, red) and *iCreER^{T2}* (iCre, green) are expressed within the same cells (arrowheads) in coronal forebrain sections of P60 *Fgfr3-iCreER^{T2}* mice (single 1 μ m optical section through the medial cortex). All sections were counter-stained with Hoechst 33258 (Hst, blue) to visualize cell nuclei. Single-labelled (arrows) and double-labelled cells (arrowheads) are indicated. Scale bars: 25 μ m.

Figure 2. Tamoxifen administration to *Fgfr3-iCreER^{T2}* transgenic mice

YFP expression was evaluated by immuno-histochemistry on 30 μ m coronal forebrain sections of P50+7 *Fgfr3-iCreER^{T2} : R26R-YFP* (a-e) and *Fgfr3-iCreER^{T2} : Z/EG* (f-i) mice. Arrows in c and e (higher-magnification images of the indicated regions in a) reveal intense YFP immunolabelling of the pial surface and the SVZ of the lateral ventricle, respectively. Reporter gene activation was very efficient and YFP-labelled cells were widespread and ubiquitous in the grey and white matter (b, d, which are higher-magnification images of the regions indicated in a). To visualize the morphology of individual cells and their processes,

we switched to *Fgfr3-iCreER^{T2} : Z/EG* mice, in which reporter gene activation was less efficient (compare panels **b** and **f** from equivalent regions as indicated in panel **a**) so labelled cells could be seen in isolation (**g-i**). At P50+7, the morphologies of GFP⁺ cells were consistent with fibrous white matter astrocytes (**g**), sub-pial astrocytes (**h**) and protoplasmic astrocytes (**i**). Sections were counterstained with Hoechst 33258 (Hst) to visualize cell nuclei. Scale bar: 0.8 mm (**a**), 40 μ m (**b-f**) or 8 μ m (**g-i**).

Figure 3. *Fgfr3-CreER^{T2}* marks astrocytes in the adult mouse forebrain.

To determine the identity of *Fgfr3*⁺ cells, 30 μ m coronal forebrain sections of P50+7 *Fgfr3-iCreER^{T2} : ZEG* (a-d, g, n, q) or *Fgfr3-iCreER^{T2} : R26R-YFP* mice (e, f, h-l, o, r) were co-immunolabelled for the reporter (GFP or YFP respectively), and a variety of neural cell type-specific markers. The vast majority of GFP/YFP⁺ cells in the medial cortex co-labelled for S100 β (a-b), but not GFAP (c). However, GFP/YFP⁺ cells co-labelled for GFAP (red) in the cortex when they were associated with blood vessels (d). GFP/YFP-labelled cell processes in contact with blood vessels co-expressed the marker Aquaporin-4 (AP4) (e).

GFP/YFP⁺ cells (green) were mostly OLIG2 negative (**f**), with rare exceptions (**g**). GFP/YFP⁺ cells and NeuN⁺ cells appeared to be largely distinct (**h**), although very rare double-positive cells were detected (**i**). In the corpus callosum the great majority of GFP/YFP⁺ cells were OLIG2-negative (**j**) but GFAP⁺ (**k**, **n-o**). Occasional cells that were GFAP-negative were observed (**l**). (**m**) The proportions of YFP⁺ cells in the corpus callosum and medial cortex of *Fgfr3-iCreER^{T2} : R26R-YFP* mice that co-express GFAP, S100 β , OLIG2 or NeuN were quantified. Not all GFAP⁺ fibrous astrocytes were GFP/YFP⁺ in the corpus callosum of P50+7 *Fgfr3-iCreER^{T2} : Z/EG* (**n**) or *Fgfr3-iCreER^{T2} : R26R-YFP* (**o**). Not all S100 β ⁺, OLIG2-negative protoplasmic astrocytes were GFP/YFP⁺ in the cortex of P50+7 *Fgfr3-iCreER^{T2} : Z/EG* (**q**) or *Fgfr3-iCreER^{T2} : R26R-YFP* (**r**). Reporter-specific recombination efficiencies (fractions of fibrous or protoplasmic astrocytes that were GFP/YFP⁺) are shown (**p**, **s**). Sections were counterstained with Hoechst 33258 (Hst) to visualize cell nuclei. Images are single confocal scans (1 μ m). Examples of single-positive (arrows) and double-immuno-positive cells (arrowheads) are indicated. Abbreviations: cortex, Ctx; corpus callosum, CC. Scale bars: 25 μ m (**a-e**, **n**, **o**, **q**, **r**), 15 μ m (**f-l**).

Figure 4. Lineage tracing of astrocytes in the corpus callosum

30 μ m coronal sections of P50+14 and P50+80 *Fgfr3-iCreER^{T2} : R26R-YFP* mouse forebrain were immunolabelled for YFP and a variety of neural cell type-specific markers. At both P50+14 (a) and P50+80 (b) almost all YFP⁺ cells (green) in the corpus callosum were GFAP⁺ fibrous astrocytes (red). A small number of YFP⁺ cells co-labelled for NG2 (c) or SOX10 (d). These data are presented quantitatively in (e). Images are single confocal scans (1 μ m). Sections were counterstained with Hoechst 33258 (Hst) to visualize cell nuclei. Single asterisk indicates $P < 0.05$. Scale bar: 10 μ m.

tamoxifen administration (P70+7) and the fraction of YFP⁺ neurospheres increased with time to ~90%. **(e)** BrdU was administered over 24 hours to P50+80 *Fgfr3-iCreER^{T2} ; R26R-YFP* mice (see Methods). Immunohistochemistry detected many YFP (green), BrdU (red) double-positive cells in the SVZ (single confocal scan; lateral ventricle marked by asterisk). A BrdU⁺, YFP⁺ cell (arrow) and a BrdU⁺, YFP-negative cell (arrowhead) are indicated. Scale bars: 40 μ m (**a, e**), 300 μ m (**b-c**).

Figure 6. Olfactory bulb interneurons inherit a recombinant *R26R-YFP* from SVZ stem cells via intermediate PSA-NCAM⁺ progenitors

It is well established that stem cells located in the adult forebrain subventricular zone (SVZ) divide to self-renew while generating progenitor cells that in turn produce PSA-NCAM⁺ neuroblasts that migrate along the rostral migratory stream (RMS) to differentiate into new olfactory bulb (OB) interneurons throughout life. Coronal forebrain sections through the SVZ, RMS and OB of P50+7 (**a-c**), P50+14, P50+80 and P50+180 (**d-f**) *Fgfr3-iCreER^{T2} : R26R-YFP* mice were immunolabelled for YFP (green) and PSA-NCAM (red). The proportions of PSA-NCAM⁺ cells that were also YFP⁺ are shown in **g**. At each time point,

coronal sections through the olfactory bulbs of *Fgfr3-iCreER^{T2} : R26R-YFP* mice were additionally immunolabelled for YFP (green) and either the pan-neuronal marker NeuN (**h-j**), or the interneuron specific markers Calretinin (Crt, **k**), Calbindin (Cb, **l**) or Tyrosine Hydroxylase (TH, **m**). At each time post-tamoxifen, the proportion of neurons that co-expressed YFP was determined (**n**). Cell counts were performed across both the granule cell layer (g) and periglomerular layer (p) of the olfactory bulb. YFP⁺ neurons of each subtype accumulated in number up until at least P50+180. High magnification single confocal scans are shown (1µm) for each region. Examples of YFP negative cells are indicated by arrowheads and YFP⁺ cells are indicated by arrows. To minimize overlap due to the high cell density, cell counts were made on single confocal scans. Sections were counterstained with Hoechst 33258 (Hst) to visualize cell nuclei. Scale bars: 25 µm (**a-f**), 20 µm (**h-m**).