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GFAPδ in radial glia and subventricular zone progenitors in the developing human cortex

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SUMMARY

A subpopulation of glial fibrillary acidic protein (GFAP)-expressing cells located along the length of the lateral ventricles in the subventricular zone (SVZ) have been identified as the multipotent neural stem cells of the adult mammalian brain. We have previously found that, in the adult human brain, a splice variant of GFAP, termed GFAPδ, was expressed specifically in these cells. To investigate whether GFAPδ is also present in the precursors of SVZ astrocytes during development and whether GFAPδ could play a role in the developmental process, we analyzed GFAPδ expression in the normal developing human cortex and in the cortex of foetuses with the migration disorder lissencephaly type II. We demonstrated for the first time that GFAPδ is specifically expressed in radial glia and SVZ neural progenitors during human brain development. Expression of GFAPδ in radial glia starts at around 13 weeks of pregnancy and disappears before birth. GFAPδ is continuously expressed in the SVZ progenitors at later gestational ages and in the postnatal brain. Co-localization with Ki67 proved that these GFAPδ-expressing cells are able to proliferate. Furthermore, we showed that the expression pattern of GFAPδ was disturbed in lissencephaly type II. Overall, these results suggest that the adult SVZ is indeed a remnant of the foetal SVZ, which develops from radial glia. Furthermore, we provide evidence that GFAPδ can distinguish resting astrocytes from proliferating SVZ progenitors.

KEY WORDS: GFAP, Radial glia, Human brain development, Neural progenitors, Lissencephaly

INTRODUCTION

A subpopulation of astrocytes in the subventricular zone (SVZ) have been identified as the multipotent neural stem cells of the adult mammalian brain (Doetsch et al., 1999; Laywell et al., 2000; Quinones-Hinojosa et al., 2006; Sanai et al., 2004). These neural stem cells express glial fibrillary acidic protein (GFAP) and are located along the length of the lateral ventricles. In the human brain, intense expression of a splice variant of GFAP, termed GFAPδ, was found at this specific location (Roelofs et al., 2005), which suggests that GFAPδ-expressing cells might be the neural stem cells of the adult human brain.

Recently, studies in rodents have shown that the adult neural stem cells in the SVZ are derived from radial glia (Bonfanti and Peretto, 2007; Merkle et al., 2004). Radial glia are bipolar cells with a soma in the ventricular zone (VZ), a short process making contact with the ventricular surface and a long radial fibre reaching the pial surface. In humans, however, there is, at present, no direct proof that adult SVZ astrocytes are derived from radial glia, although they have similar features. First, a number of SVZ neural progenitors maintain direct contact with the ventricles, like radial glia (Sanai et al., 2004; Tramontin et al., 2003). Second, neural progenitors from the SVZ migrate a long distance to the olfactory bulb in the adult rodent brain (Lois and Alvarez-Buylla, 1994); however, this is still under debate for the human brain (Curtis et al., 2007; Sanai et al., 2004; Sanai et al., 2007). This could be compared to the migration of neural progenitors from their site of birth in the VZ and SVZ to their final destination in the developing brain. Third, like radial glia, adult human SVZ neural stem cells express vimentin, nestin and GFAP (Sanai et al., 2004).

The development of the layered structure of the cortex can be nicely monitored in sections of the developing cerebral cortex from human foetuses of various ages throughout gestation. In the course of early development, cortical neurons emerge from the radial glia in the VZ, which are direct descendants of the primitive neuroepithelium. The classical view is that radial glia are astrocyte precursor cells important for guiding neuronal migration (Bystron et al., 2008; Rakic, 1971). However, recent studies have shown that they can also act as neural stem cells, being capable of divisions that lead to the genesis of astrocytes, neurons (Götz et al., 2002; Hartfuss et al., 2001; Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002) and oligodendrocytes (Merkle et al., 2004). Radial glia express the intermediate filament (IF) proteins vimentin and nestin, and also, in primates, GFAP. As development proceeds, a second proliferative zone becomes discernable between the VZ and the cell-sparse intermediate zone, that is, the SVZ. The number of rapidly proliferating progenitor cells in the SVZ grows significantly during the last trimester, in parallel with a reduction in the number of cells in the VZ. Finally, the VZ will disappear before birth and the SVZ will persist into adulthood (Tramontin et al., 2003).

A disturbance of the layered development of the human brain is observed in the developmental neuronal migration disorder type II lissencephaly, which is neuropathologically characterized by loss of sulci and broadening gyri, with often irregular appearance of the brain surface in affected regions (cobblestone appearance). This type of cortical dysplasia results from a failure of the arrest of radial neuronal migration due to defects in the integrity of the pial/glial

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barrier. Consequential histopathological microscopic features are represented by a highly disorganized cerebral cortex structure, with focal absence of a glia limitans, disturbance of radial fibres, and neuronal and glial heterotopias in the leptomeninges (Squier, 1993).

In the current study, we explored the developmental path of adult SVZ astrocytes in the normal developing human brain and in brains of type II lissencephalic foetuses by analyzing GFAPδ expression. We hypothesized that, if the presence of GFAPδ is important for the development of SVZ neural stem cells, it would also be expressed in its precursors during development. We furthermore expect that the localization of these precursors is disturbed in lissencephalic brains due to a disruption of radial glia and that this can be observed by studying the GFAPδ-expressing cells. In addition, continuous expression of GFAPδ in this lineage would provide evidence that the adult human SVZ astrocytes originate from radial glia.

MATERIALS AND METHODS

Human brain material

The subjects included in this study were obtained from the brain collections of the Department of Neuropathology of the Academic Medical Center, University of Amsterdam, The Netherlands and the Service Histologie-Embryologie-Cytogénétique Hôpital Necker-Enfants Malades, Paris, France. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. The expression of GFAPδ was evaluated during brain development; the following ages were included: gestational week (gw) 9, 10, 13, 16, 17, 20, 22, 23, 25, 29, 31, 36 and 40, based on last menstrual period and ultrasound scanning. The tissue was obtained from spontaneous or medically induced abortions, with appropriate maternal written consent for brain autopsy. We also obtained normal-appearing control cortex/white matter at autopsy from three young cases (3 months, 7 months and 8.5 years), without a history of seizures or other neurological diseases. In addition, we obtained tissue from three cases with type II lissencephaly of 21, 22 and 23 gw. All autopsies were performed within 12 hours of death. More details of the human brain material used are summarized in Table S1 in the supplementary material.

Tissue preparation

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 μm and mounted on organosilane-coated slides (Sigma, St Louis, MO, USA), and used for immunocytochemical staining as described below.

Immunocytochemical analysis

For single labelling, paraffin-embedded sections were deparaffinized, rehydrated and incubated for 20 min in 0.3% H_2O_2 diluted in methanol to quench the endogenous peroxidase activity. Antigen retrieval was performed by incubation for 10 min at 121°C in citrate buffer (0.01 M, pH 6.0) in a pressure cooker. Sections were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 and 4 mM Na_2HPO_4, pH 7.4) and incubated for 30 min in 10% normal goat serum (Harlan Sera-Lab, Loughborough, Leicestershire, UK). After incubation with polyclonal rabbit pan-GFAP, which recognizes all GFAP isoforms (DAKO, Glostrup, Denmark; 1:4000), and GFAPδ [bleeding 100501; 1:500 (Roelofs et al., 2005)] primary antibodies in PBS overnight at 4°C, we washed the sections in PBS. We then used the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3′-diaminobenzidine (DAB; Sigma) as a chromogen to visualize the antibodies. Sections were counterstained with haematoxylin, dehydrated and coverslipped.

To determine more specifically the cell type of GFAPδ-expressing cells in the developing brain, we performed several co-localization studies. We combined GFAPδ with monoclonal mouse antibodies against vimentin (clone V9; DAKO; 1:1000), a known marker of radial glia in the VZ during human brain development (Honig et al., 1996), nestin (MAB5326, Chemicon; 1:200), a marker of both a subpopulation of radial glia (Zecevic, 2004) and SVZ neural progenitors (Lendahl et al., 1990), Ki67 (clone MIB-1, DAKO; 1:200), a marker of proliferation (Scholzen and Gerdes, 2000), and with polyclonal rabbit antibody against Sox2 (AB5603, Chemicon; 1:200), a transcription factor important for the maintenance of neural stem cells (Graham et al., 2003). For double labelling with polyclonal goat antibody against the C terminus of GFAPα, which could, in principle, also detect other low-expressed GFAP isoforms, for example, GFAPΔ135 (Hol et al., 2003) (GFAP C-term; Santa Cruz Biotechnology; 1:200), we blocked with normal swine serum instead of normal goat serum. After incubation overnight with the primary antibodies at 4°C, sections were incubated for two hours at room temperature with Alexa Fluor 568-conjugated anti-rabbit IgG and Alexa Fluor® 488 anti-mouse or anti-goat IgG (1:100, Molecular Probes, The Netherlands). Sections were mounted with Vectashield containing DAPI (which targets DNA in the cell nucleus; blue emission) and analyzed using a laser scanning confocal microscope (Leica TCS Sp2, Wetzlar, Germany). Sections incubated without the primary antibody or with pre-immune serum (for GFAPδ) were essentially blank, except for the outer layer of the VZ/ependymal layer, which, in some cases, appeared a bit darker when treated with the pre-immune serum.

GFAP plasmids and transfections

To obtain recombinant protein samples for western blotting, expression plasmids pcDNA3-GFAPα and pcDNA3-GFAPδ (Roelofs et al., 2005) were transfected separately into SH-SY5Y neuroblastoma cells, which do not express GFAP endogenously. These cells were cultured in high-glucose DMEM, supplemented with 10% heat-inactivated foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C with 5% CO_2. The cell culture medium was refreshed two hours before lipofectamine (Invitrogen) transfection with pcDNA3-GFAPα or pcDNA3-GFAPδ plasmids, according to the manufacturer's protocol.

Western blot

Protein was isolated from GFAPα- and GFAPδ-transfected SH-SY5Y cells by homogenization with lysis buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The samples were dissolved in 2× loading buffer (2×: 100 mM Tris, 4% SDS, 20% glycerol, 200 mM DTT, 0.006% bromophenol blue) and boiled for five minutes. Subsequently, they were run on a 7.5% SDS-PAGE gel and blotted semi-dry on nitrocellulose. Blots were probed overnight with polyclonal rabbit pan-GFAP (DAKO; 1:20 000), GFAPδ [bleeding 100501; 1:500 (Roelofs et al., 2005)] or polyclonal goat C-terminal GFAPα (GFAP C-term; Santa Cruz Biotechnology; 1:200) antibodies diluted in Supermix (0.05 M Tris, 0.9% NaCl, 0.25% gelatin and 0.5% Triton X-100, pH 7.4) antibodies. The next day, the blots were washed with TBS-T (TBS; 100 mM Tris-HCl pH 7.4, 150 mM NaCl, with 0.2% tween-20) and incubated with secondary antibody anti-rabbit IRDye800 or anti-goat IRDye800 (1:5000; Rockland Immunochemicals, Gilbertsville, USA) in Supermix for one hour at room temperature. After three washes in TBS, bands were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

RESULTS

Different GFAP antibodies recognize specific GFAP isoforms

A western blot analysis of recombinant GFAPα or GFAPδ protein was performed to verify the specificity of the different GFAP antibodies used in this study. As indicated in a schematic picture (Fig. 1A), the epitope of the pan-GFAP antibody is not known. The GFAP C-term and GFAPδ antibodies are directed to a sequence in the C terminus of, respectively, GFAPα and GFAPδ. At approximately 55 kDa, protein bands for both GFAPα and GFAPδ protein were detected with the pan-GFAP antibody, whereas the GFAPδ antibody specifically detected the GFAPδ protein, as was previously shown (Roelofs et al., 2005). The antibody directed against the C terminus of GFAPα only detected GFAPα recombinant protein, not GFAPδ (Fig. 1B).

DEVELOPMENT

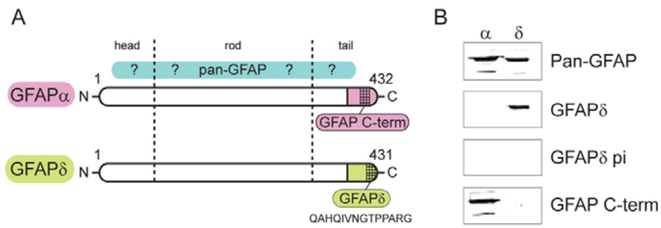

Fig. 1. Western blot analysis determines the specificity of different GFAP antibodies. (**A**) A schematic picture of the GFAPα and GFAPδ proteins, indicating the epitopes of the different GFAP antibodies used in this study. The specific epitope for the pan-GFAP antibody is not known. The GFAP C-term antibody recognizes an unknown sequence in the C terminus of GFAPα, which is different from the C terminus of GFAPδ. The GFAPδ antibody was raised against a specific sequence (QAHQIVNGTPPARG) in the C terminus of GFAPδ. (**B**) GFAPα and GFAPδ recombinant protein were both recognized by the pan-GFAP antibody at approximately 55 kDa. The GFAPδ antibody specifically recognized the GFAPδ protein at approximately 55 kDa, but not GFAPα. Additionally, the pre-immune serum of the GFAPδ-immunized rabbit (GFAPδ pi) was tested and showed no immunoreactivity. The antibody raised against a peptide sequence at the C terminus of human GFAP solely recognized GFAPα protein at approximately 55 kDa.

GFAPδ expression during human brain development visualizes the development of the neurogenic astrocytic ribbon

GFAPδ expression was not observed in brain sections at 10 gw and younger (Fig. 2A). In addition, the pan-GFAP antibody failed to show any GFAP expression in adjacent sections (Fig. 2B). The first GFAPδ expression was detected at 13 gw in cells lining the VZ along the lateral ventricles (Fig. 2C). These cells had a bipolar morphology, with somata in the VZ, short processes towards the ventricles and longer fibres extending into the brain parenchyma. These radial-glia-like cells were also intensely stained by the pan-GFAP antibody, which more clearly labelled the long fibres extending from the VZ into the brain parenchyma (Fig. 2D). Around 17 gw, the SVZ clearly expressed GFAPδ and possibly other GFAP isoforms as well, because we also observed pan-GFAP expression (Fig. 2E,F). The VZ cell fibres, which were also still visible at this stage in development, seemed to extend through the SVZ cell layer, which contained more round and unipolar cells. GFAPδ expression at this stage was clearly less pronounced than that of pan-GFAP, pointing to stronger expression of other GFAP isoforms. This difference became even more evident at older ages, when additional transitional profiles of radial glia appeared and cells became more differentiated (Fig. 2G-J). At 22 gw, the VZ cells still expressed GFAPδ, as did the cells in the SVZ (Fig. 2G). However, with the pan-GFAP antibody, much more intense labelling was detected and also more cells outside the SVZ were positive for pan-GFAP (Fig. 2H), whereas very little GFAPδ expression was found in cells outside the VZ and SVZ. At the perinatal age of 36 gw, the VZ cells were no longer present and GFAPδ expression was limited to the SVZ and some processes in the ependymal layer (Fig. 2I), whereas pan-GFAP expression was also found throughout the rest of the brain (Fig. 2J). This GFAPδ expression pattern is similar to that observed postnatally (not shown) and in the adult brain (Roelofs et al., 2005).

GFAPδ-positive cells are a subpopulation of all GFAP-expressing cells

Although several GFAP isoforms, including GFAPδ, were expressed in the developing VZ and SVZ, superficial layers of the developing cortex lacked GFAPδ expression, but did clearly express other

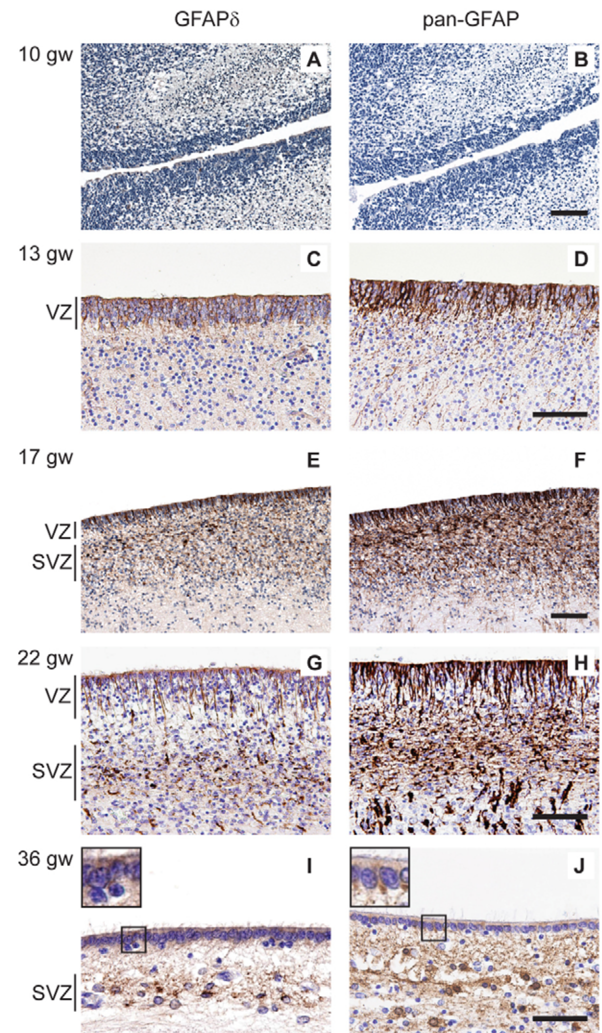

Fig. 2. Development of the GFAPδ astrocytic ribbon during human brain development. (**A**,**B**) At gestational week 10 (10 gw), no expression was found for either GFAPδ (A) or pan-GFAP (B). (**C-J**) At 13 gw, GFAPδ expression was found exclusively in the VZ (C), where it continued to be expressed at 17 and 22 gw (E,G). However, at these time points, GFAPδ expression also became clear in the SVZ (E,G). GFAPδ expression in a ribbon of cells in the SVZ remained visible at 36 gw (I) and even until adulthood (not shown). Some GFAPδ-expressing processes were also detected in the ependymal layer (I, magnified box). Pan-GFAP expression was also visible at 13 gw (D), although compared to GFAPδ expression, it was more intense in both the VZ and SVZ throughout development (D,F,H,J). From 22 gw, pan-GFAP expression also became clear outside the SVZ, more distant from the ventricle (H,J) and in the ependymal layer (J, magnified box). The expression pattern of pan-GFAP continued to be more extensive than that of GFAPδ. Haematoxylin counterstain shows blue nuclei. Scale bars: 100 μm in B,D,F,H; 50 μm in J.

GFAP isoforms. The intermediate zone (IZ) and the cortical plate (CP), including the marginal zone (MZ) and the subplate (SP), did not express GFAPδ (Fig. 3A,C), but the pan-GFAP antibody clearly showed staining in the IZ (Fig. 3B), the CP and MZ (Fig. 3D).

To show that the GFAPδ-expressing cells in the VZ and SVZ express other GFAP isoforms alongside GFAPδ, we studied double labelling of the GFAPδ antibody with the GFAP C-term antibody, which detects GFAPα, the predominant GFAP isoform, but not

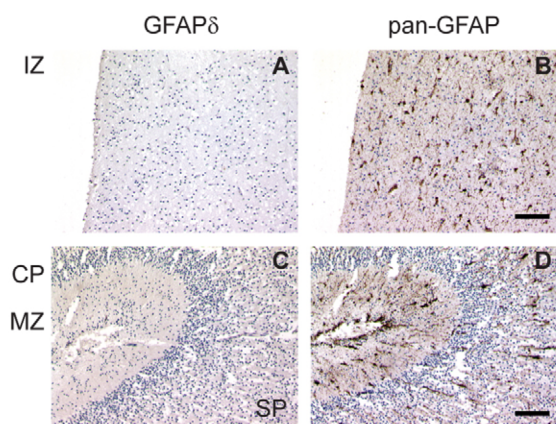

Fig. 3. Pan-GFAP immunoreactivity reaches further than that of GFAPδ. At 25 gw, there was no GFAPδ expression in the IZ, the CP, the MZ and the SP (**A**,**C**). In these areas, however, pan-GFAP was clearly expressed (**B**,**D**). Haematoxylin counterstain shows blue nuclei. Scale bars: 100 μm.

GFAPδ, as was shown in Fig. 1. At 25 gw, both GFAP C-term (Fig. 4A) and GFAPδ (Fig. 4B) were expressed in VZ and SVZ cells. All cells in the VZ showed double labelling of the two GFAP isoform antibodies (Fig. 4C,D), indicating that the IFs in these cells contained at least both GFAPα and GFAPδ. The composition of the IFs clearly varied between cells, as some cells showed more GFAP C-term expression (Fig. 4D, arrowhead), whereas others showed more GFAPδ expression (Fig. 4D, arrow). In the SVZ, all GFAPδ-expressing cells also expressed GFAP C-term; however, in this area some cells were also visible that were only GFAP C-term positive (Fig. 4C, arrowheads). At 36 gw, GFAPδ expression was limited to a number of cells in the SVZ that consistently co-expressed GFAP C-term (Fig. 4F-H, asterisks). Compared to GFAPδ, GFAP C-term was more widely expressed in all cells and processes in the SVZ (Fig. 4E,F).

GFAPδ is expressed in radial glia of the VZ and astrocytes of the SVZ

At 13 gw, when GFAPδ is initially expressed in the VZ (Fig. 2C, Fig. 5A,D), vimentin expression was clearly found in radial glia, with long radial fibres extending from the VZ towards the pial surface (Fig. 5B). GFAPδ and vimentin expression co-localized in the soma of these cells in the VZ and in the short process extending toward the lateral ventricle, but GFAPδ expression was not detected in the entire radial fibre (Fig. 5C). Furthermore, nestin was also expressed in the VZ radial glia cells (Fig. 5E) and was entirely co-localized with GFAPδ (Fig. 5F). At 25 gw, a clear subdivision could be made between VZ and SVZ cells, which both expressed GFAPδ (Fig. 5G,J). At this age, vimentin was no longer expressed in the long radial fibres, but could only be detected in the VZ. The cells in the SVZ did not express vimentin (Fig. 5H); hence, co-localization of GFAPδ and vimentin was limited to the radial glia in the SVZ (Fig. 5I).

In contrast, nestin was present in the SVZ in addition to the VZ at this stage of development (Fig. 5K). All cells in the VZ co-expressed nestin and GFAPδ, as did the majority of cells in the SVZ (Fig. 5L). Just a few cells in the SVZ only expressed nestin and lacked GFAPδ (Fig. 5L, boxed areas). Most cells showed co-localization of the different IF proteins, but the expression levels and the subcellular location of the proteins varied among cells. GFAPδ is more highly expressed around the cell nucleus and vimentin is found more in the extensions of the cell. This results in cells showing either more green or more red fluorescence, even though they co-express both proteins.

Together these results showed that vimentin was expressed in radial glia and nestin was expressed in both radial glia and SVZ neural progenitors during human brain development. Interestingly, co-localization with GFAPδ demonstrated for the first time that this GFAP isoform is also specifically expressed in radial glia and SVZ neural progenitors.

In addition, we double labelled several sections with GFAPδ and Sox2. At 25 gw, approximately 40% of the SVZ cells expressed Sox2, but only a few clearly expressed GFAPδ (see Fig. S1A, arrow, in the supplementary material). VZ radial glia were almost all stained by Sox2, although nuclear staining was rare. A few GFAPδ-expressing radial glia were found that contained a Sox2-positive nucleus, generally when the nucleus did not border the ventricle (see Fig. S1B, arrow, in the supplementary material).

GFAPδ is expressed in proliferating cells

A characteristic of neural progenitors is their proliferative capacity. To determine whether GFAPδ-expressing cells indeed also proliferate, we studied the co-localization of GFAPδ with Ki67, a nuclear marker of proliferating cells, at 13 (Fig. 6A-D) and 25 gw (Fig. 6E-H). At 13 gw, approximately 15% of all nuclei in the VZ

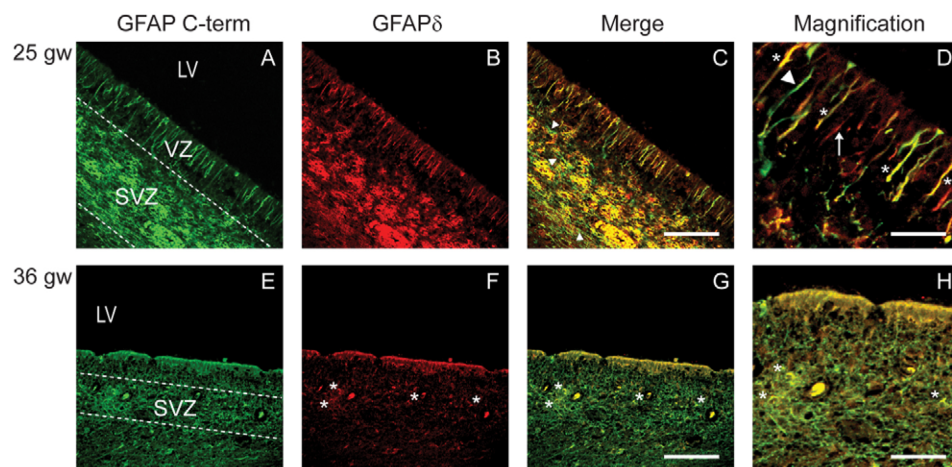

Fig. 4. GFAPδ co-localization with GFAP C-term. GFAP C-term was expressed in the VZ and SVZ at 25 gw (**A**), as was GFAPδ (**B**). GFAPδ and GFAP C-term co-localized in most cells (**C**); only some cells in the SVZ solely expressed GFAP C-term (C, arrowheads). Higher magnification of the VZ showed fibres that clearly co-expressed GFAPδ and GFAP C-term (**D**, asterisks), and fibres that showed more GFAPδ expression (D, arrow) or more GFAP C-term expression (D, arrowhead). At 36 gw, GFAP C-term was widely expressed (**E**), whereas GFAPδ was only expressed in a number of cells in the SVZ (**F**, asterisks) that also expressed GFAP C-term (**G**,**H**, asterisks). Scale bars: 100 μm (C,G); 25 μm (D); 50 μm (H). LV, lateral ventricle.

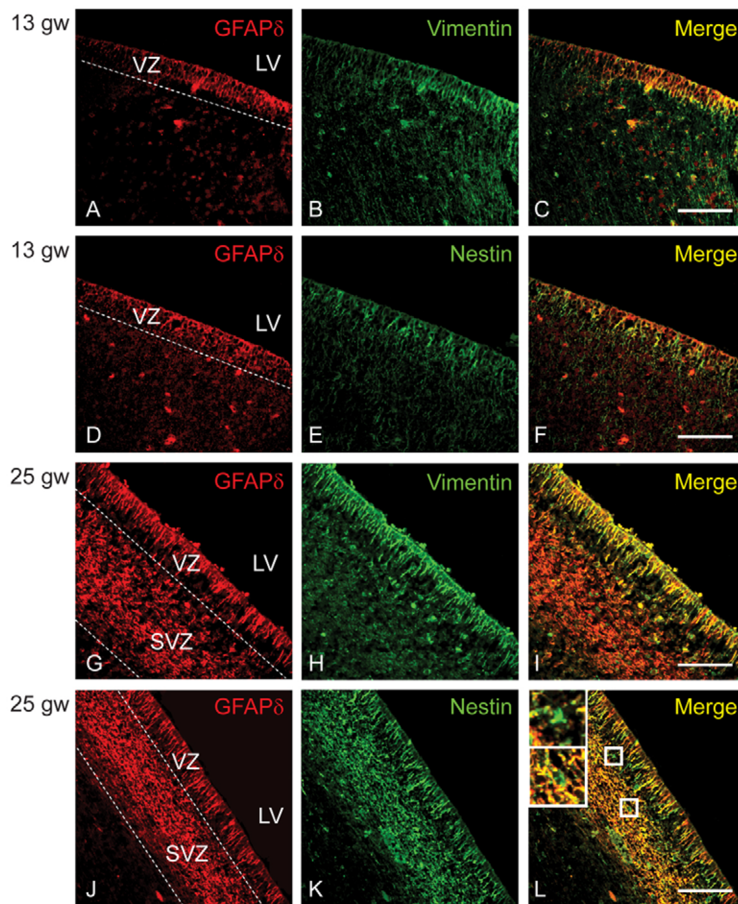

Fig. 5. GFAPδ co-localization with vimentin and nestin. GFAPδ expression was found in the VZ at 13 gw (**A**,**D**), similar to expression of the radial glia marker vimentin (**B**). These two proteins co-localized in the soma of the radial glial cells in the VZ, although not in the extended radial fibres, which only expressed vimentin (**C**). These GFAPδ-expressing radial glial cells also co-expressed nestin (**E**,**F**). Vimentin was not expressed in the SVZ at 25 gw, only in the VZ (**H**). GFAPδ at 25 gw was expressed in both the VZ and the SVZ (**G**,**J**), but only co-localized with vimentin in the VZ (**I**). At 25 gw, nestin labelled both the SVZ neural progenitors and VZ radial glia (**K**), and all cells that expressed nestin also expressed GFAPδ (**L**). Scale bars: 100 μm. LV, lateral ventricle.

were Ki67 positive (Fig. 6B) and over 90% of these Ki67-positive nuclei were enclosed by a GFAPδ-positive cell body (Fig. 6C), which was clearly evident at higher magnification (Fig. 6D). At 25 gw, the number of Ki67-positive nuclei in the VZ decreased to approximately 5%, but more Ki67-positive nuclei were found further away from the ventricle in the SVZ (Fig. 6F). Nearly all Ki67-positive nuclei in the VZ clearly belonged to a GFAPδ-positive radial glial cell, although the lack of GFAPδ directly around the nucleus hampers the interpretation of the expression results for some of the cells. These nuclei were found either bordering the ventricle or in the middle of the VZ (Fig. 6G,H). Although to a lesser extent, Ki67-positive nuclei were also found in GFAPδ-positive cells in the SVZ (Fig. 6G, arrow). In addition, we detected expression of GFAPδ and GFAPα, the major form of GFAP, in human foetal neurospheres (data not shown). Concisely, GFAPδ-expressing cells are indeed proliferating cells.

GFAPδ expression in transitional profiles of radial glia around the SVZ

Besides the VZ radial glia and SVZ neural progenitors, several GFAPδ-expressing cells were also found more distant from the ventricle on the border of the SVZ and the IZ. These cells lacked a typical radial glial phenotype and are called transition cells, because it is probable that these cells are radial glia undergoing a transformation into a more differentiated cell type (deAzevedo et al., 2003). At 25 gw, an area close to the SVZ showed GFAPδ expression in bipolar and unipolar cells that appeared to be migrating away from the SVZ (Fig. 7A). Unipolar cells were found with radial fibres directed both to the ventricular surface and to the pial surface (Fig. 7B). Among those cells, we also observed several GFAPδ-expressing multipolar cells with a more stellate morphology, like early differentiated astrocytes (Fig. 7C). Furthermore, GFAPδ expression was detected in various long, slender fibres and in thicker bipolar transition cells (Fig. 7D,E). These last transitional profiles were also found to express Ki67, which indicates that these cells are also proliferative (Fig. 7F,G). In the MZ and the SP, where fully differentiated astrocytes reside, GFAPδ protein was never present.

Distinctive GFAPδ expression in type II lissencephaly

To explore whether the localization of neural progenitors is disturbed in lissencephaly type II, we studied the expression of GFAPδ in these donors. In all three cases of type II lissencephaly, the cerebral cortex was disorganized, with lack of horizontal lamination in some areas and variable thickness. The pial barrier (Fig. 8, arrowheads) was clearly disrupted, which is associated with overmigration of neurons and glia into the leptomeningeal tissue (Fig. 8, white arrows). Pan-GFAP immunostaining (Fig. 8A) revealed the presence of regular astrocytes in the cortical plate and the glia limitans underlying the intact parts of the pia (arrowheads). In addition, pan-GFAP-expressing astrocytes were found in the leptomeningeal tissue (black arrows). Interestingly, GFAPδ-expressing cells were also present in the leptomeninges (Fig. 8B,C, black arrows), implying a disturbed localization of the neural progenitors.

DISCUSSION

In the present study, we describe for the first time the expression of GFAPδ in the developing human brain. We show that this specific splice variant of GFAP is expressed in proliferating radial glial cells in the VZ at 13 gw and in neural progenitors of the SVZ at later

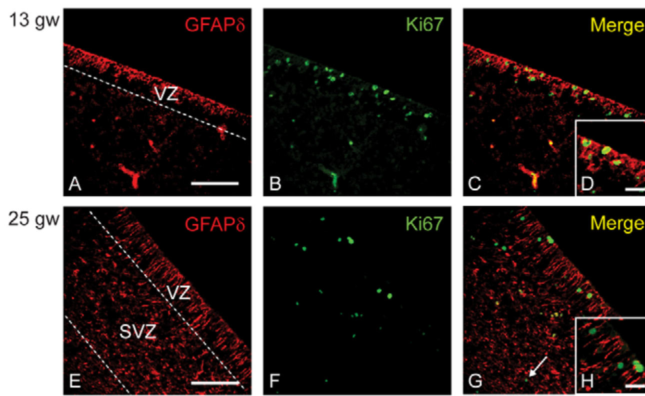

Fig. 6. GFAPδ expression in proliferating cells. (**A-H**) Expression of GFAPδ protein and proliferation marker Ki67 at 13 gw (A-D) and 25 gw (E-H). At 13 gw, many Ki67-expressing nuclei were present in radial glia of the VZ (B). Almost all Ki67-expressing nuclei were surrounded by GFAPδ-expressing cell bodies and processes (C); this was more evident at higher magnification (D). At 25 gw, the number of Ki67-positive nuclei in the VZ was reduced (F), but the number of positive nuclei located in the SVZ had increased. It was generally clear that a Ki67-positive nucleus belonged to a GFAPδ-expressing radial glial cell in the VZ (G,H). Also, some GFAPδ-expressing cells in the SVZ contained a Ki67-positive nucleus (G, arrow). Scale bars: 100 μm in A,E; 25 μm in D,H.

gestational ages. Furthermore, we show that the normal position of GFAPδ-expressing neural progenitors during development is disturbed in lissencephaly type II. GFAPδ expression was previously found in a subpopulation of astrocytes in the SVZ of the adult human brain (Roelofs et al., 2005). The location of these cells corresponds to a ribbon of astrocytes lining the lateral ventricles of the adult human brain that contains neural stem cells (Sanai et al., 2004). By showing continuous expression of GFAPδ in the SVZ of the developing human cortex, we conclude that the adult SVZ is a remnant of the foetal SVZ, which in turn develops from VZ radial glial cells. These findings support the function of GFAPδ in neural progenitors during development and in adulthood.

The earliest time point in development when we found clear GFAPδ and pan-GFAP expression was at 13 gw. In brain tissue of 10-week-old foetuses or younger, no immunoreactivity was found with any of the GFAP antibodies. Unfortunately, we did not have access to good-quality material from 11- or 12-week-old human foetuses, which would have enabled us to determine initial GFAPδ expression more precisely. Several studies have reported that, as in monkeys (Levitt and Rakic, 1980), GFAP staining appeared in radial glia of the human brain at 9 to 12 gw (Antanitus et al., 1976; deAzevedo et al., 2003; Honig et al., 1996; Messam et al., 2002; Simonati et al., 1997; Stagaard and Mollgard, 1989). One study described a small population of GFAP-expressing cells in the premordium plexiform layer by 6 gw, at the onset of corticogenesis (Honig et al., 1996). However, other studies have reported little or no GFAP staining until 14-25 weeks (Aquino et al., 1996; Sarnat, 1992; Sasaki et al., 1988). In most reports, as in our study, the number of immunopositive cells increases with gestational age (Aquino et al., 1996; deAzevedo et al., 2003; Honig et al., 1996; Messam et al., 2002) and is much higher during the second half of gestation. The differences between some of these studies and ours might be explained by differences in the brain areas investigated, antibodies used and the staining methods, which have significantly improved over the years.

Co-localization of GFAPδ with GFAP C-term showed that GFAPδ-expressing cells also express other isoforms of GFAP (Fig. 4). Most probably, these cells express the predominant isoform, GFAPα, although it is not possible to exclude the presence of other isoforms in these cells by immunocytochemistry. Moreover, in the SVZ and beyond, cells were found expressing GFAP C-term, but not GFAPδ. Together with the finding that the pan-GFAP antibody stains astrocytes in more superficial layers of the cortex compared to GFAPδ (Fig. 3), we demonstrated that GFAPδ is expressed in a subpopulation of all GFAP-expressing cells in the developing human brain. More differentiated or matured astrocytes in other areas of the developing brain lacked GFAPδ expression, but might express other isoforms of GFAP (Blechingberg et al., 2007; Hol et al., 2003). These results are in accordance with the study by Pollard et al. showing that GFAPδ levels decrease following in vitro differentiation (Pollard et al., 2009).

To prove that the GFAPδ-expressing cells in foetal brains were indeed radial glia and neural progenitors, we performed co-localization studies with several cell type markers. Vimentin, which is an IF protein, like GFAP, is known to be present in radial glial cells of the VZ during human brain development (Honig et al., 1996). Furthermore, it has been reported that a subpopulation of radial glial cells express the IF protein nestin (Zecevic, 2004). Nestin is a commonly used stem cell marker and is also expressed in neuroepithelial cells early in development (Tohyama et al., 1992). We confirmed the vimentin expression in radial glia and the nestin expression in both radial glia and SVZ neural progenitors during human brain development. More importantly, by showing co-localization of these markers with GFAPδ, we demonstrated for the first time that this GFAP isoform is also specifically expressed in

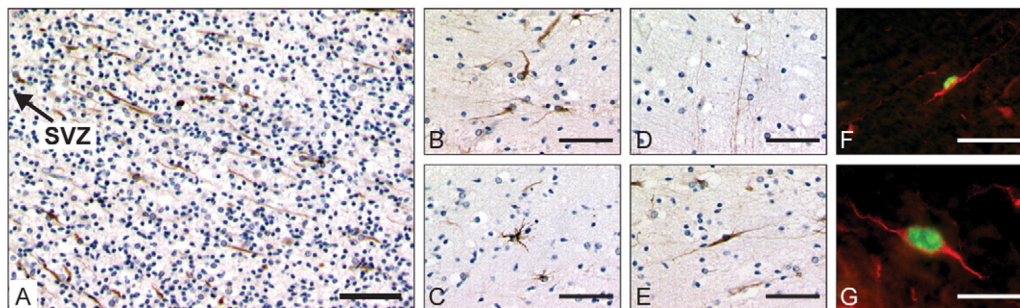

Fig. 7. GFAPδ expression in transitional profiles of radial glia at 25 gw. (**A**) A stream of parallel GFAPδ-expressing cells were present on the border of the SVZ and the IZ. The arrow indicates the direction of the SVZ. (**B-E**) GFAPδ expression was found in monopolar cells (B), multipolar cells (C), and bipolar cells with slender (D) and thick (E) fibres. (**F**,**G**) Several bipolar GFAPδ-expressing cells were found with a Ki67-positive nucleus. Scale bars: 100 μm in A; 50 μm in B-F; 20 μm in G.

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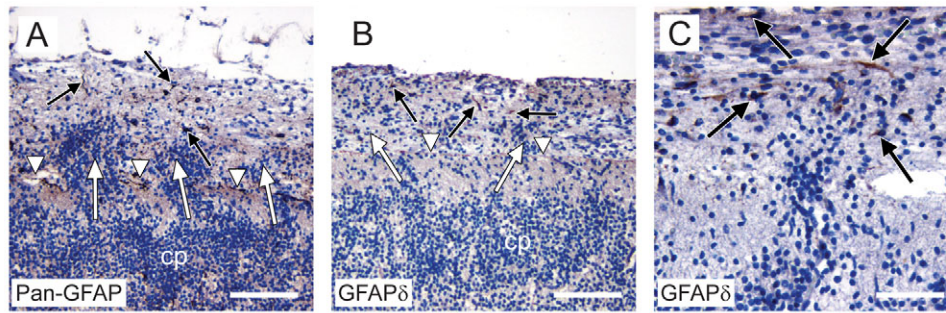

Fig. 8. Pan-GFAP and GFAPδ expression in type II lissencephaly. Example of pan-GFAP (**A**) and GFAPδ (**B**,**C**) staining in a female type II lissencephaly donor of 22 gw with a POMGnT1 gene mutation. The pial barrier was still intact in some places (white arrowheads), but where it was disrupted, neurons and astrocytes had migrated into the leptomeningeal tissue (white arrows). Pan-GFAP immunostaining revealed astrocytes in the cortical plate (CP), the glia limitans, and also in the leptomeninges (A, black arrows). GFAPδ immunostaining was also detected in astrocytes of the leptomeninges (B, black arrows). At higher magnification, clear GFAPδ-positive cells were detected around the protrusion of migrating cells and deeper into the leptomeningeal tissue (C, black arrows). Scale bars: 50 μm in A,B; 20 μm in C.

radial glial cells and SVZ neural progenitors (Fig. 5). In addition, we proved that these cells are capable of dividing. Double labelling with the proliferation marker Ki67, present during all active phases of the cell cycle (Scholzen and Gerdes, 2000; Tramontin et al., 2003), showed that all Ki67-positive nuclei in the VZ clearly belonged to a GFAPδ-positive radial glial cell (Fig. 6). These nuclei were found either bordering the ventricle or in the middle of the VZ (Fig. 6G,H), which is an indication of nuclear translocation, a process that occurs in radial glia and has been considered an essential component of neuronal migration (Berry and Rogers, 1965; Tramontin et al., 2003). Furthermore, we showed co-expression of GFAPδ and Sox2 (see Fig. S1 in the supplementary material). Sox2 is a transcription factor essential for the maintenance of neural progenitor characteristics during development (Graham et al., 2003) and was also shown to be expressed in GFAP-positive neural progenitors in the adult human SVZ (Baer et al., 2007). In summary, GFAPδ-expressing cells in the developing human brain are proliferating radial glial cells and SVZ neural progenitors.

In our previous studies, we showed that GFAPδ is expressed in balloon cells in focal cortical dysplasia and hemimegalencephaly, and in giant cells in tuberous sclerosis complex (Lamparello et al., 2007; Martinian et al., 2009). Because at that time, GFAPδ was only described in SVZ astrocytes (Roelofs et al., 2005), an area in which neural progenitors are located, we put forward the idea that the GFAPδ subpopulation of balloon cells retains a progenitor phenotype or represents an influx of newly generated astrocytes from the SVZ. Several studies provided evidence that balloon cells in focal cortical dysplasia retain an embryonic phenotype and are derived from radial glial cells (Crino et al., 1997; Mizuguchi et al., 2002; Sisodiya et al., 2002; Thom et al., 2005). The fact that we found GFAPδ expression in radial glia during brain development supports this view.

In this study, we found GFAPδ not only in the germinal zones, but also in areas further away from these zones, where several transitional profiles of radial glia reside (deAzevedo et al., 2003). These profiles are considered to represent the transformation of radial glial cells into astrocytes. However, this is only based on static observations and it is not known whether these transition cells could also transform into neurons. In particular areas of our tissue, transitional profiles of radial glial cells seemed to be migrating away from the VZ and SVZ (Fig. 7). These findings imply that GFAPδ could be present in migrating cells and possibly plays a role in migration.

Besides the spatial variability, the intensity of GFAPδ immunoreactivity in radial glial and progenitor cells was also variable among different areas of the VZ and SVZ. This suggests heterogeneity between radial glial cells and SVZ cells, as has been proposed before (Pinto and Götz, 2007). The amount of GFAPδ expression proportionate to GFAPα expression might indicate a functional difference in the IF network among progenitor cells. This assumption is based on previous studies that show changes in IF network formation with different levels of GFAPδ expression in vitro (Nielsen and Jorgensen, 2004; Perng et al., 2008; Roelofs et al., 2005). We have reported before that, in human astrocyte cell lines and spinal cord samples, the levels of GFAPδ are about 10% of the levels of GFAPα, a ratio that ensures a build-up of an intact IF network (Perng et al., 2008). The level of GFAPδ in a cell might be an indication of the flexibility of the cytoskeleton, which would suggest a potential role for GFAPδ in radial glia and progenitor cells in regulating cell motility and migration, as was previously reported for GFAP in astrocytes (Lepekhin et al., 2001). However, in vivo functional data have not yet been obtained for this splice variant.

The presence of GFAPδ can also affect the interaction with IF-associated proteins, such as αB-crystallin, and can result in activation of cell signalling molecules, such as phosphorylation of JNK (Perng et al., 2008). Moreover, GFAPδ can also interact with the homologous transmembrane proteins presenilin 1 (PS1) and presenilin 2 (PS2) (Nielsen et al., 2002), which are associated with genetic forms of Alzheimer's disease (Bertram and Tanzi, 2008). Interestingly, PS1 has a key role in morphogenesis of the developing cortex. It is required for neuronal migration and cortical lamination, and can modulate radial glia development and neuronal differentiation via Notch signalling (Gaiano et al., 2000; Handler et al., 2000; Louvi et al., 2004; Schuurmans and Guillemot, 2002; Shen et al., 1997). In addition, PS1 deficiency in mice leads to cortical dysplasia with characteristics that are very similar to those of human type II lissencephaly (Hartmann et al., 1999). Our finding of GFAPδ expression in the leptomeninges in lissencephaly type II suggests that neural progenitor migration is disrupted and unrestrained, and that these cells fail to make the transition into a differentiated cell type before they reach the cortical plate. Another explanation for the unusual pattern of GFAPδ expression might be that lissencephaly induces proliferation or differentiation of a non-neurogenic astrocyte subtype that is distinguished by GFAPδ expression.

Whether the interaction of GFAPδ with presenilin could be involved in this disturbed development requires further investigation. Regulation of neuronal migration by PS1 showed striking similarities to regulation by cyclin-dependent kinase 5 (Cdk5) (Ohshima et al., 2002). Cdk5 is associated with the IF protein nestin (Sahlgren et al., 2003), which is co-expressed with GFAPδ in the developing cortex (Fig. 5F,L).

In conclusion, we show that GFAPδ is a protein that might have important functions in radial glia and neural progenitors in the human developing cortex. The interaction of GFAPδ with proteins involved in development and cell signalling implies that this specific GFAP isoform has a function in human brain development. Further research is needed to find out the exact role of GFAPδ in neural stem cells, their migration and the commitment of their progeny.

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Competing interests statement

The authors declare no competing financial interests

Supplementary material

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