Astrocytes in development, aging and disease: starring GFAP

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Longterm quiescent cells in the aged human subventricular neurogenic system specifically express GFAPδ

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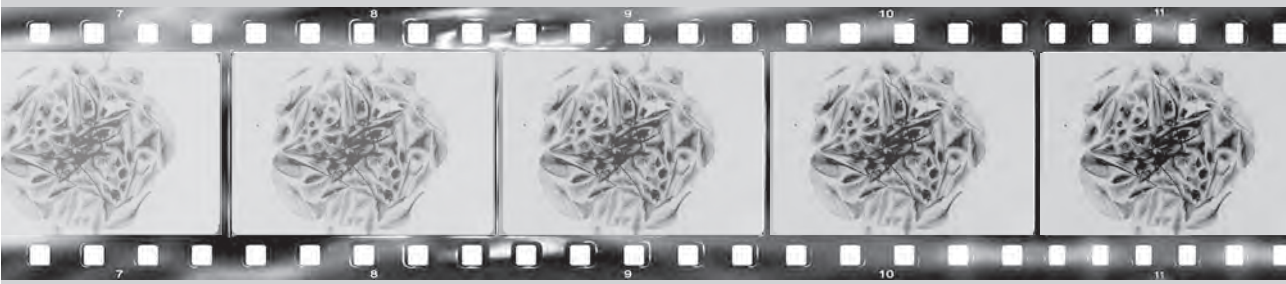


Abstract

A main neurogenic niche in the adult human brain is the subventricular zone (SVZ). Recent data suggest that the progenitors that are born in the human SVZ migrate via the rostral migratory stream (RMS) towards the olfactory bulb (OB), similar to what has been observed in other mammals. A subpopulation of astrocytes in the SVZ specifically expresses an assembly-compromised isoform of the intermediate filament protein glial fibrillary acidic protein (GFAPδ). To further define the phenotype of these GFAPδ expressing cells and to determine whether these cells are present throughout the human subventricular neurogenic system, we analyzed SVZ, RMS and OB sections of 14 aged brain donors (ages 74-93). GFAPδ was expressed in the SVZ along the ventricle, in the RMS and in the OB. The GFAPδ cells in the SVZ co-expressed the neural stem cell (NSC) marker nestin and the cell proliferation markers PCNA and Mcm2. Furthermore, BrdU retention was found in GFAPδ positive cells in the SVZ. In the RMS, GFAPδ was expressed in the glial net surrounding the neuroblasts. In the OB, GFAPδ positive cells co-expressed PCNA. We also showed that GFAPδ cells are present in neurosphere cultures that were derived from SVZ precursors, isolated postmortem from four brain donors (ages 63-91). Taken together, our findings show that GFAPδ is expressed in an astrocytic subpopulation in the SVZ, the RMS and the OB. Importantly, we provide the first evidence that GFAPδ is specifically expressed in longterm quiescent cells in the human SVZ, which are reminiscent of NSCs.

Introduction

The adult human brain contains two regions where continuous neurogenesis takes place, i.e. the subventricular zone (SVZ) (Sanai et al. 2004; Quinones-Hinojosa et al. 2006) and the subgranular zone in the hippocampal dentate gyrus (Eriksson et al. 1998; Roy et al. 2000). In particular, the SVZ maintains a level of plasticity into adulthood and can respond to certain neurological conditions, such as stroke and Huntington's disease, by increasing the number of neuron forming precursor cells that can potentially replace damaged neurons. In animal models of brain trauma, disease and stroke, the SVZ responds by producing new precursor cells, which migrate to the damaged areas and form new regionally specific neurons (Parent et al. 2002; Cooper & Isacson 2004). In the human brain, a similar response seems to occur after ischemia (Macas et al. 2006). The SVZ can react in this way because it is closely situated to the striatum, which is affected in Parkinson's disease and Huntington's disease and it receives nutrients from the cerebrospinal fluid in the lateral ventricle (for review, see (Curtis et al. 2007a)). One of the key questions

that remain to be answered is which cells in the SVZ are responsible for ultimately producing the new cells in the human SVZ.

The composition of the human SVZ is different from that of the more studied rodent species because of the presence of a hypocellular gap between the ependymal layer and the dense astrocytic ribbon, the latter of which contains the neural precursors (Quinones-Hinojosa *et al.* 2006). There are indications that a subpopulation of astrocytes in this ribbon, the neurogenic astrocytes, proliferate *in vivo* and behave as multipotent precursor cells *in vitro* (Sanai *et al.* 2004), implying that SVZ astrocytes of the adult human brain indeed are neural stem cells (NSCs). Within the SVZ, three major cell types exist, one of which is the neurogenic astrocyte known as B-cell (Doetsch *et al.* 1997), which is a slowly proliferating cell that asymmetrically divides to form a pool of fast-dividing neural progenitor cells, the C-cells. Type C-cells in turn differentiate into type A-cells, called neuroblasts, which migrate through the rostral migratory stream (RMS) into the olfactory bulb (OB), where they differentiate into new interneurons (Curtis *et al.* 2007b). The existence of an RMS in the human brain, long thought absent, was recently described by Curtis *et al.*, (2007b). Recently, we have shown that NSCs are still present in the SVZ of elderly subjects, including cases with neurodegenerative diseases, such as Alzheimer's disease (Leonard *et al.* 2009).

NSCs express certain intermediate filament (IF) proteins and some reports claim the specificity of these for labeling NSCs. IFs are highly versatile cytoskeletal structures that have a major function in cell signalling and migration (Lepekhin *et al.* 2001; Pallari & Eriksson 2006; Herrmann *et al.* 2007). One IF expressed in NSCs is nestin, widely regarded as specific for NSCs (for review, see (Gilyarov 2008)), but conflicting reports reveal nestin expression in both reactive and mature astrocytes (Lin *et al.* 1995; Gu *et al.* 2002). Our laboratory has been interested in specific IF expression as they relate to NSCs. To this end, our previous work has revealed that SVZ astrocytes in the human brain express the IF protein glial fibrillary acidic protein δ (GFAPδ) (Roelofs *et al.* 2005), one of the seven splice variants of GFAP (Hol *et al.* 2003; Quinlan *et al.* 2007), the main IF protein in astrocytes (Eng *et al.* 2000)). Moreover, we recently have described that GFAPδ is expressed in radial glia and SVZ progenitors of the human fetal brain (Middeldorp *et al.* in press), substantiating our earlier findings that this specific GFAP isoform is a marker for NSC. GFAPδ (also named GFAPε (Nielsen *et al.* 2002)) has a unique 41 amino acid carboxy-terminus. This unique tail domain endows the protein with several properties distinct from GFAPα, which are likely to have important functional consequences for the IF network formed in the SVZ astrocytes. Firstly, GFAPδ is an assembly-compromised form of GFAP (Nielsen *et al.* 2002; Roelofs *et al.* 2005; Perng *et al.* 2008) and its presence in the IF network changes the composition of the

IF network and its associated proteins, such as αB-crystallin (Perng *et al.* 2008). The network can tolerate up to 10% of the GFAPδ isoform, which is approximately the amount found in the central nervous system (Perng *et al.* 2008). A second property of GFAPδ is its ability to interact with presenilins (Nielsen *et al.* 2002), which are essential partners in the gamma-secretase complex. This complex cleaves the transmembrane proteins Notch and amyloid precursor protein (APP) inside the membrane region and therefore is critical for Notch and APP signalling (Selkoe & Kopan 2003). Notch signalling is required for maintenance of NSC populations in both the developing and adult brain (Alexson *et al.*, 2006) and Notch1 expression can be found in SVZ astrocytes and neuroblasts in the adult rodent brain (Givogri *et al.* 2006). Thirdly and finally, the induced expression of GFAPδ *in vitro* results in an increased phosphorylation of Jnk (Perng *et al.* 2008). In the developing brain, Jnk is involved in NSC proliferation (Wang *et al.* 2007), migration (Mizuno *et al.* 2005), and differentiation (Kim *et al.* 2007).

Localization of GFAPδ and its ability to modulate signalling pathways important for NSCs suggest that GFAPδ is expressed by NSCs in the adult SVZ. Here we have studied the expression of GFAPδ in the SVZ, RMS and OB in the adult human brain. We provide evidence that the GFAPδ expressing cells may indeed be quiescent adult NSCs, as they are present throughout the human adult neurogenic system, in a subpopulation of proliferating astrocytes in the SVZ, and in neurosphere cultures isolated from postmortem adult human SVZ material.

Materials and methods

Postmortem human brain material

Tissue from the SVZ, RMS, and OB was obtained from the Netherlands Brain Bank (NBB; Amsterdam, The Netherlands) and the department of Anatomy and Neurosciences (ANW), VU University Medical Centre (Amsterdam, The Netherlands). The NBB and ANW perform brain autopsies with regularly short postmortem intervals, and the brain donors have given informed consent for using the tissue and for accessing the extensive neuropathological and clinical information for scientific research, in compliance with ethical and legal guidelines (Huitinga *et al.* 2008). Clinico-pathological information of all donors can be found in Table 1.

* SVZ material

SVZ tissue for immunohistochemistry was sampled from three standardized areas in the brain as depicted in Fig. 1A. SVZ1 was dissected out at the level of the most frontal part of the caudate nucleus; SVZ2 came from the area beneath the cingulate gyrus; SVZ3 contained the most posterior part of the lateral ventricle.

Table 1 Clinico-pathological data of the brain donors

NBB nr.	Area studied	Sex	Age (yrs)	Diagnosis	Braak stage (Tau/amyloid)	PMD (h:min)	pH CSF	Brain weight (g)	Cause of death
05-019	OB	m	74	Non-demented control	3 C	05:00	6.70	1125	Bronchus carcinoma
05-061	OB	f	93	Non-demented control	2 O	05:50	--	1145	Cachexia
05-065	OB & RMS	f	93	Non-demented control	1 A	04:25	7.30	1223	Heart failure
05-083	OB	f	85	Non-demented control	1 B	05:00	6.72	1257	Multi organ failure after a ruptured abdominal aneurysm
06-080	OB & RMS	f	89	Non-demented control	2 B	06:25	6.46	1210	Old age, possible ruptured abdominal aneurysm
07-007	SVZ & OB	m	84	Non-demented control	1 A	05:35	6.98	1457	Heart failure
07-014	SVZ & OB	m	86	Non-demented control	2 B	04:00	--	1250	Respiratory insufficiency
07-032	SVZ	f	87	Non-demented control	3 O	07:20	6.24	1140	Cachexia and dehydration
07-046	SVZ	m	89	Non-demented control	1 B	09:20	6.40	1285	Sudden death
07-075	SVZ	f	82	Non-demented control	2 O	05:10	6.64	1210	Pneumonia by hemothorax
06-002	RMS	f	84	Parkinson's disease	2 B	07:25	6.85	1244	Unknown
06-049	RMS	f	84	Non-demented control	1 O	04:45	6.26	1179	Heart failure
ANW228	RMS	m	91	Non-demented control	2 B	<24:00	--	--	Heart failure
ANW237	RMS	m	82	Non-demented control	2 B	<24:00	--	--	Old age, dehydration
08-018	Cultured SVZ	f	89	Control with vascular encephelopathy	4 B	03:30	6.49	1220	Renal insufficiency
08-029	Cultured SVZ	m	84	Alzheimer's disease	6 C	08:05	5.95	1345	Shock by hematemesis
08-076	Cultured SVZ	f	91	Depression	3 C	05:20	6.53	1233	Cachexia and dehydration by pneumonia and renal insufficiency
08-078	Cultured SVZ	m	63	Frontotemporal dementia	0 O	06:05	6.27	1327	Cachexia and dehydration by end stage dementia

NBB = Netherlands Brain Bank; SVZ = subventricular zone; RMS = rostral migratory stream; OB = olfactory bulb; m = male; f = female; Braak stage is a scale for Alzheimer pathology, scoring tau (Braak and Braak, 1991) and amyloid (Thal et al., 2000) pathology; PMD = post-mortem delay; CSF = cerebrospinal fluid; pH CSF indicates the agonal state of the donor (Ravid et al., 1992).

In SVZ1, both white and grey matter could be found aligning the SVZ, while in SVZ2 and SVZ3 only white matter was seen. The donor brain tissue for this part of our study was selected using the following criteria: the clinical history of donors was free from any neurological disease or neuropsychiatric disorder; their brains did not have an Alzheimer Braak score higher than 3 or significant Parkinson's disease pathology. Furthermore, we excluded donors who had recently used medication that might affect neurogenesis, i.e. anti-mitotics and anti-depressants.

For neurosphere cultures, SVZ tissue was freshly isolated from the anterior horn of the SVZ. As we were restricted to the material that we could obtain from the NBB and the fact that the full neuropathological report is not available at the starting point of culturing, we included in this part of the study also patients with neurological and neuropsychiatric diseases.

* **BrdU material**

Striatal tissue containing the SVZ was used from two donors who had received BrdU for diagnostic purposes as previously described (Eriksson *et al.* 1998). Case 1 was female and received BrdU 129 days before death. Case 2 was also female and died 1693 days after BrdU administration.

* **RMS and OB material**

Tissue of the human forebrain comprising the region surrounding the anterior horn of the lateral ventricle, frontal part of the caudate nucleus, gyrus rectus, substantia perforata anterior and OT, was sampled to study the RMS. Samples of the OT and OB were collected and processed for immunohistochemistry as well. The control donors for analysis of the RMS and OB were selected as for the SVZ. All tissue was immersion-fixed in phosphate-buffered 4% paraformaldehyde (PFA; pH 7.4) for 4 weeks before processing.

Immunostaining

Immunostaining was performed according to a fairly standard protocol, described in full in the supplemental data. Briefly, epitope retrieval was performed in a steamer and a serum blocking step was used before incubating with antibody (supplementary Table S1) overnight. The antibodies were visualized using biotinylated secondary antibodies, ABC, and DAB, or fluorescently labeled secondary antibodies.

Human postmortem neurospheres cultures

Neurosphere cultures were initiated as described previously (Leonard *et al.* 2009). The obtained microglia-poor cell pellet was taken up in serum-free medium (SFM, Neurobasal medium, 1% B27, 0.5% N2, 1% Glutamax, 1% P/S, Hepes (all Invitrogen-Life Technologies, Carlsbad, CA, USA), 1% Ultraglutamin (Cambrex Corporation, East Rutherford, NJ, USA), 5 µg/ml heparin (Sigma-Aldrich, St. Louis, MO, USA),

20 ng/ml epidermal growth factor (EGF), and 20 ng/ml fibroblast growth factor (FGF; both Tebu-Bio, Paris, France) and plated in a 12 well plate. For differentiation, spheres were centrifuged and replated in complete Dulbecco's Modified Eagle medium (DMEM; containing 10% fetal calf serum, 1% P/S, Hepes; all Invitrogen) on 12mm poly-L-lysine coated glass coverslips. For quantitative real-time polymerase chain reaction (qPCR), single neurospheres were isolated and taken up in 100 µl Trizol (Invitrogen). RNA was isolated according to the manufacturer's protocol, with addition of glycogen to facilitate RNA precipitation. A DNase (Invitrogen) step was used to degrade genomic DNA contamination. cDNA synthesis and qPCR for GFAPδ were performed as previously described (Perng *et al.* 2008).

For immunostaining, the neurospheres were fixed for 20 min in 3.7% formaldehyde solution (Sigma) in phosphate buffered saline (PBS; 50 mM potassium phosphate, 150 mM NaCl; pH 7.2), after which they were embedded in TissueTek (Bayer, Leverkusen, Germany) for cryosectioning and mounted on Superfrost plus slides. Differentiated cells were fixed with formaldehyde solution in PBS on coverslips. Then, the 10µm thick cryosections or coverslips were washed in TBS and incubated with antibodies (see supplementary Table S1) in TBS-BSA for 16-24 hours at 4°C. After three rinses with TBS, secondary antibodies in TBS-BSA were applied for 1 hour at RT. Then, the sections or coverslips were rinsed three times and embedded in Mowiol.

Image acquisition and analysis

Images of single immunostainings were obtained on an AxioSkop microscope (Zeiss, Oberkochen, Germany) with Neoplanfluor objectives, using a Sony XC77 black and white camera (Sony, San Diego, California) and ImagePro software (MediaCybernetics, Bethesda, USA). Images of immunofluorescent staining were obtained on an AxioPlan 2 microscope (Zeiss) with Planapochromat objectives, using an Evolution QEi black and white camera (MediaCybernetics) and ImagePro software. Confocal images were obtained on a Zeiss LSM 510 Meta Confocal Microscope (Zeiss).

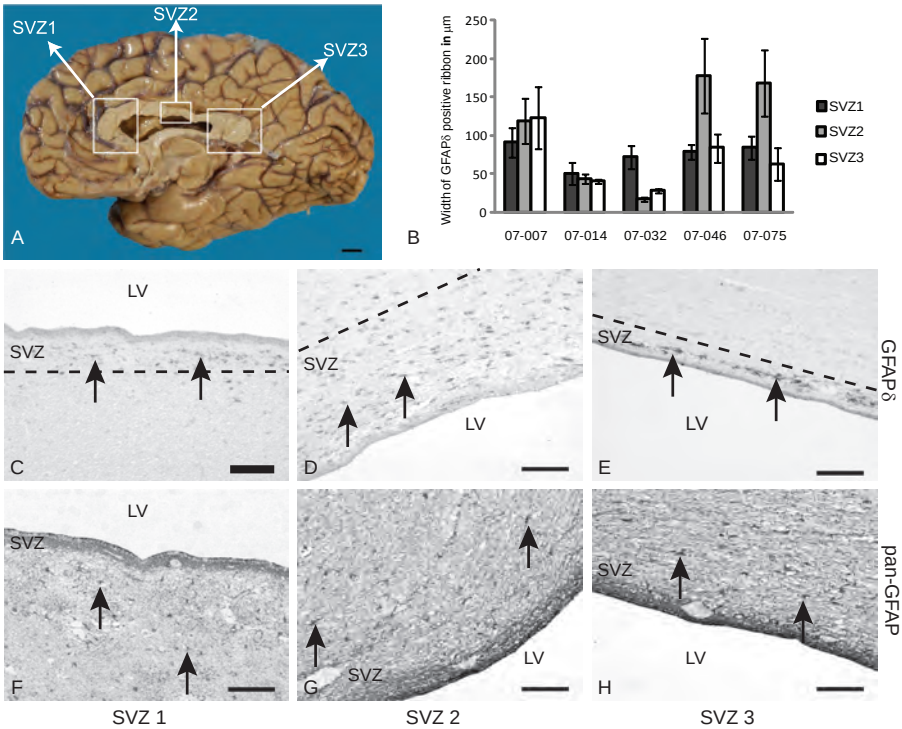
→ **Figure 1 GFAPδ in the aged human SVZ.** (A) Schematic location of SVZ tissue samples as collected from the Netherlands Brain Bank (NBB). SVZ1 was taken out at the level of the most frontal part of the caudate nucleus; SVZ2 came from the area beneath the cingulate gyrus; SVZ3 contained the most posterior part of the lateral ventricle. (B) Quantification of the width of the GFAPδ ribbon in frontal sections throughout the SVZ of 5 different donors. (C-E) Examples of GFAPδ staining (arrows) throughout the SVZ of a control brain (NBB 07-075). (F-H) pan-GFAP staining in the same SVZ areas; staining can be found in the parenchyma (arrows) and in the SVZ (arrowheads). Scale bars indicate 1cm in A), 100µm in C-H); data in (B) are represented as mean values ± SEM, n=5 measurements per SVZ region; LV = lateral ventricle; dashed line indicates border of GFAPδ positive ribbon. Picture A) Courtesy of NBB and photo service Pathology department VUMC, NL.

For the quantification of the width of the GFAPδ ribbon, low magnification images were taken of the whole SVZ present in one section. In this image, an area of interest (AOI) was drawn around the SVZ. In this AOI, 5 images were captured at high magnification in a randomized way. In the high magnification image, again an AOI was drawn, this time around the GFAPδ positive area. The average width of this AOI was measured and used as the width of GFAPδ staining.

Results

GFAPδ is robustly expressed in an astrocytic ribbon of variable width along the full length of the SVZ

We have previously described that GFAPδ is expressed in the SVZ along several brain structures and subpial layers of the adult human brain (Roelofs *et al.* 2005). In the current study, we have systematically studied its expression pattern throughout the SVZ of five aged control brains at three standardized locations (see Fig. 1A, and Experimental procedures). When staining these three areas for GFAPδ, we observed a band of immunostaining in the SVZ throughout the three sampled locations. The cells in this band often had an elongated morphology, parallel to the ependymal lining of the SVZ. The width of the immunoreactive band, which



ranged from 20 to 200μm (Fig. 1B), was not constant within the three SVZ areas of one donor and showed also large variability among donors. We have performed a correlation analysis between the variability and several characteristics of the donors, such as age, sex, postmortem delay and medication used, and found no significant correlations. Fig. 1 C-E shows a typical example of the strong variability of the thickness of the band of GFAPδ expressing astrocytes throughout the SVZ of one donor. The SVZ regions of the same donor stained for all GFAP isoforms revealed many more GFAP positive cells and encompassed labeling in the caudate nucleus, SVZ and in the hypocellular gap (Fig. 1 F-H). These data substantiate our earlier findings that GFAPδ is expressed in a subset of astrocytes in the SVZ, forming a ribbon. In addition, we provide a first report on the large variation in the width of the astrocytic ribbon within and between donors, and in the quantity of GFAPδ positive cells in the SVZ.

GFAPδ is expressed in immature, proliferative SVZ astrocytes with stem cell characteristics

To establish the specific phenotype of the cells in the astrocytic ribbon that express GFAPδ, we applied a double-immunolabeling technique using various astrocyte-specific markers in the three SVZ areas. Results of confocal analysis are shown in Fig. 2; images showing anatomical localization are shown in supplemental Fig. S1. GFAPδ was expressed in a subpopulation of astrocytes that also expressed the astrocyte marker GFAPα (Fig. 2A), as shown by clear co-localization with an antibody specific for the carboxy-terminus of GFAP. The immature astrocyte marker vimentin, an intermediate filament protein, also co-localized with GFAPδ (Fig. 2B). To the contrary, the calcium binding protein S100B, and the enzyme glutamine synthetase (GS), a marker of fully differentiated astrocytes, were not expressed in GFAPδ cells. S100B was expressed in a distinct cell population in the SVZ (Fig. 2C), most likely representing more mature niche astrocytes; expression of GS was only found in cells of the parenchyma and not in the SVZ (Fig. 2D). GFAPδ was not expressed by postmitotic neurons, as was shown by the absence of co-localization with NeuN (data not shown).

To investigate whether GFAPδ cells in the aged SVZ are NSCs, we studied the co-expression of GFAPδ with proliferation markers and markers for NSCs. Results of confocal analysis are shown in Fig. 3; images showing anatomical localization are shown in supplemental Fig. 2.We found that all GFAPδ positive cells also express the proliferation marker proliferating cell nuclear antigen (PCNA; Fig. 3A), the mitosis marker minichromosome maintenance complex component 2 (Mcm2; Fig. 3B) and the transcription factor Sox2 (Fig. 3C). The latter marker showed staining in both cell nuclei and in cytoplasm, which has been described before for the human brain (Baer *et al.* 2007). The phosphorylated form of the histone H3 protein (pHH3) was

not expressed in GFAPδ positive cells (Fig. 3D), and also no double-labeling with Ki-67, another proliferation marker, was seen (Fig. 3E); in fact very few pHH3 and Ki-67 positive cells were present in the SVZ, which is in agreement with earlier reports (Kukekov *et al.* 1999). Attempts to co-localize GFAPδ with CD133 were unsuccessful in this paraffin-embedded material.

We also studied co-localization with the thymidine analogue BrdU, which labels dividing cells in the S-phase. In one of the brains treated with BrdU, many BrdU positive nuclei were found in the SVZ near the caudate nucleus, many of which were GFAPδ positive (Fig. 3F). Besides the SVZ astrocytes, many fibres around BrdU positive ependymal cells were GFAPδ positive. Deeper into the brain parenchyma, BrdU positive nuclei were scarce. In the second human BrdU case, much less BrdU labeling was found, and there was a corresponding reduction in the number of double-positive cells. In the second BrdU case the time from BrdU injection to postmortem examination was protracted compared to the first case. Our data so far demonstrate that GFAPδ positive cells are proliferative, which is one indication that these cells are indeed NSCs.

Subsequently, we studied whether the cells co-express markers for different stages of stem cell differentiation. We observed considerable co-expression between GFAPδ and nestin (B cells; Fig. 3G). However, this pattern was variable throughout the SVZ; we observed regions with almost complete overlap between the stainings, as well as locations with minimal overlap. We also observed that GFAPδ positive cells only

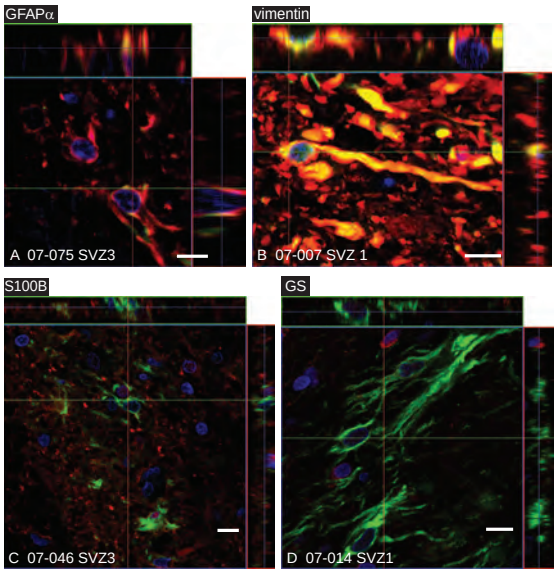


Figure 2 Fluorescent double immunostaining of GFAPδ with astrocyte markers in the SVZ. GFAPδ staining is shown in green, the respective astrocyte markers in red. A) GFAPδ is expressed in a subpopulation of GFAPα positive cells. (B) Vimentin expression was found mainly in the SVZ, in all GFAPδ positive cells. (C) S100B is expressed throughout the tissue, in the SVZ there is no co-expression with GFAPδ. (D) Glutamine synthetase was expressed only in the parenchyma, not in the SVZ. Blue staining is Topro staining for cell nuclei; scale bar indicates 5µm in A and B; 10µm in C and D; individual NBB numbers of the cases are indicated in the left bottom corner.

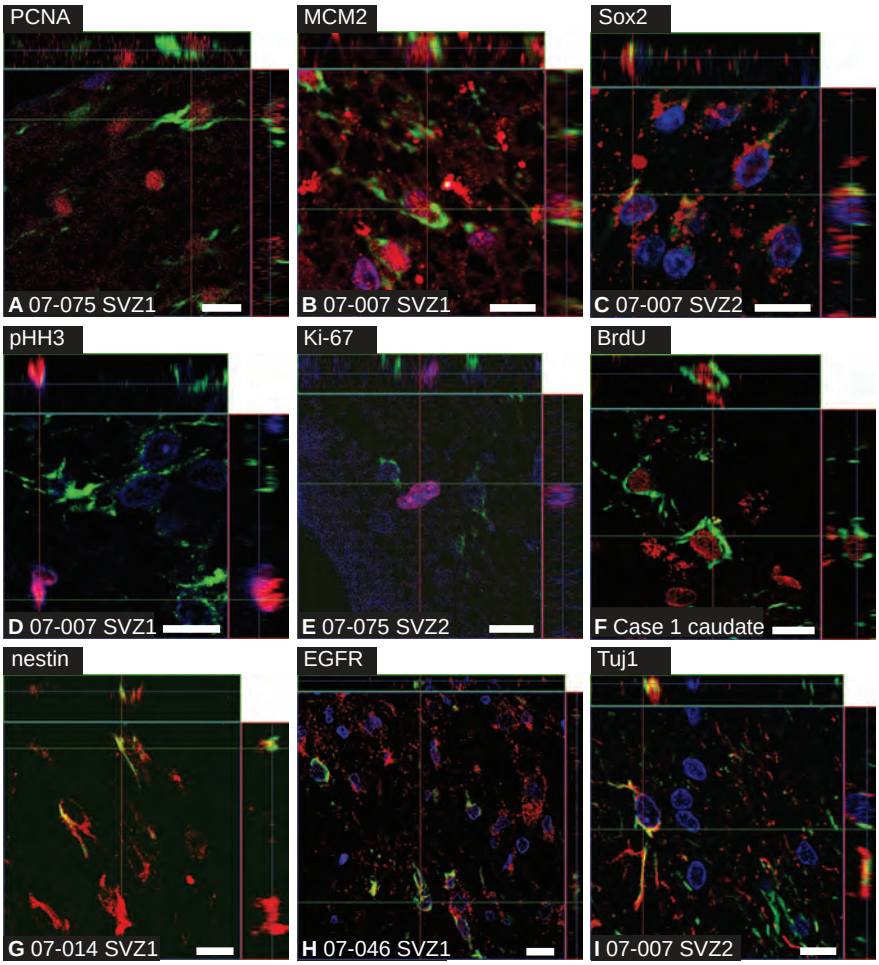


Figure 3 Fluorescent double immunostaining of GFAPδ (green) with proliferation and stem cell markers (red) in the SVZ of different control donors. (A) PCNA expression is high in the SVZ; most GFAPδ positive cells express PCNA. (B) Most GFAPδ positive cells are Mcm2 positive. (C) Sox2 is expressed in nuclei and cytoplasm of GFAPδ positive cells. (D) pHH3 positive cells were extremely rare and did not express GFAPδ. (E) Ki-67 positive cells could only be found occasionally and were not GFAPδ positive. (F) BrdU labeling was found in many GFAPδ positive cells. (G) In double immunostainings for nestin and GFAPδ, cells could be found with both proteins and with the single proteins. (H) EGF-R was hardly ever found in GFAPδ expressing cells. (I) Rarely, single cells could be found expressing GFAPδ and βIII-tubulin. Blue staining is Topro staining for cell nuclei; scale bars indicate 10μm in all; individual NBB numbers of the cases are indicated in the left bottom corner.

rarely expressed the C-cell marker EGF-R (Fig. 3H) or the A-cell marker βIII-tubulin (Fig. 3I). Attempts to co-localize GFAPδ with PSA-NCAM or DCX, using different antibodies (as mentioned in supplementary table S1), were unsuccessful.

GFAPδ is expressed in the glial net surrounding the RMS

In 2007, the first details emerged about the existence of the RMS in the human brain, although organized differently than in mice (Curtis *et al.* 2007b). Furthermore, Alonso *et al.* showed that astrocytes in the rodent RMS are neurogenic (Alonso *et al.* 2008). Therefore, we examined the aged human RMS for the presence of GFAPδ. Indeed, we found GFAPδ to be highly expressed throughout the RMS (Fig. 4). In fact, GFAPδ is a very reliable and robust marker of the RMS in the human brain. In sagittal sections taken from the RMS, the dorsal and rostral limb of the RMS were visible in a number of sections (Fig. 4A-C). The dorsal limb of the RMS contained a broad band of GFAPδ immunoreactive stellate-shaped cells with cell bodies that were about 8-10mm in diameter and had many long processes. Fewer GFAPδ immunoreactive cells were found in the rostral limb of the RMS. There was large variation in the number of GFAPδ immunoreactive cells in the RMS within and between donors.

In frontal sections from the striatum, which we studied for different purposes, the dorsal limb of the RMS was visible. In these sections, we observed that in double immunostaining of GFAPδ with PCNA, PCNA was highly expressed in the centre of the stream of the RMS, whereas GFAPδ positive astrocytes were predominantly found at the border of this stream (Fig. 4D). No co-localization was found between GFAPδ and PCNA in the dorsal limb of the RMS (Fig. 4D).

GFAPδ is expressed in proliferative cells in the olfactory bulb

The OB and olfactory tract (OT) were also highly enriched in GFAPδ positive cells (Fig. 5). In the centre of the OT, the GFAPδ positive cells seemed to be organized in streams. Most GFAPδ positive cells were however located in the outer layers of the OT (Fig. 5B). In the OB, GFAPδ positive cells were primarily found in the olfactory nerve layer and glomerular layer (Fig. 5C and D). Few cells were also localized within the external plexiform layer and granule cell layer. Double-labeling of GFAPδ with PCNA in the OB (Fig. 5E and supplemental Fig. 3E) showed that the GFAPδ cells were proliferative. Since the cells were negative for βIII-tubulin (Fig. 5F and supplemental Fig. 3F), we conclude that the GFAPδ positive cells were not neuroblasts. In contrast to the SVZ, we very rarely observed co-expression of vimentin and GFAPδ (Fig. 5G and supplemental Fig. 3G).

GFAPδ is expressed in postmortem human adult neurospheres

To obtain further evidence that GFAPδ is expressed in adult NSCs, we aimed at determining whether GFAPδ is expressed in neurospheres derived from the SVZ of human adult brain. Although the neurosphere assay has some limitations (Reynolds & Rietze 2005), it has long been used to assess whether a particular area of the CNS harbours stem cells. We were able to culture neurospheres from postmortem adult human material using a protocol we described recently (Leonard *et al.* 2009) (Fig. 6A). We isolated NSCs from each donor that came to autopsy and this was successful for 80% of the cases. As previously described, we could obtain neurospheres (Fig. 6A)

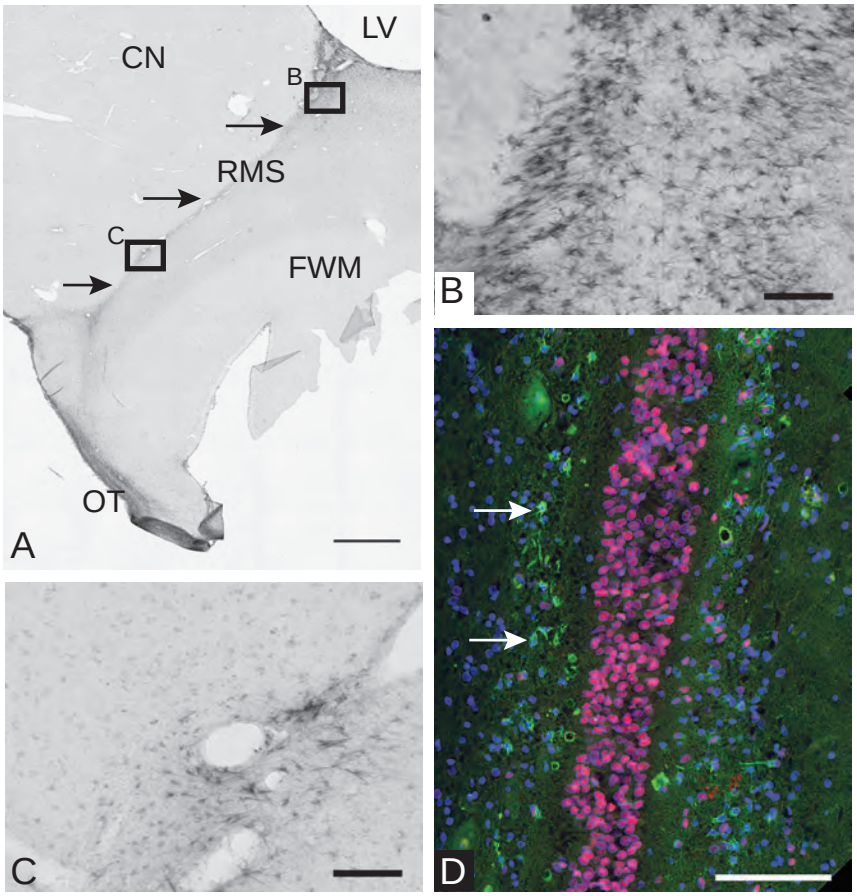


Figure 4 GFAPδ expression in the rostral migratory stream. (A) Overview of the RMS (arrows) of case ANW228 from the SVZ to the OT in a sagittal section. Boxed areas are enlarged in (B) and (C). (D) Fluorescent immunostaining for PCNA (red) and GFAPδ (green) (arrows) in a frontal section of the dorsal limb of the RMS of case NBB 06-002. Blue staining is Hoechst staining for cell nuclei. LV = lateral ventricle, CN = caudate nucleus, FWM = frontal white matter, RMS = rostral migratory stream, OT = olfactory tract; scale bars indicate 2mm in (A), 0.1mm in B-D.

from elderly donors with and without neurodegenerative diseases. We found that GFAPδ is indeed expressed in proliferative and multipotent neurospheres. In all the cultures that we stained for GFAPδ, we could find immunoreactivity (Fig. 6B-E), overlapping the expression of GFAPα (Fig. 6B), nestin (Fig. 6C), and vimentin (Fig. 6D). In the neurospheres, expression of GFAPδ and the early neuronal marker βIII-tubulin was found mainly in distinct cell populations (Fig. 6E). Detailed analysis of co-labeling using confocal microscopy, showing co-localization with GFAPα, nestin and vimentin, but not βIII-tubulin can be found in supplemental Fig. 4. We confirmed the expression of GFAPδ at the RNA level by qPCR in different individual

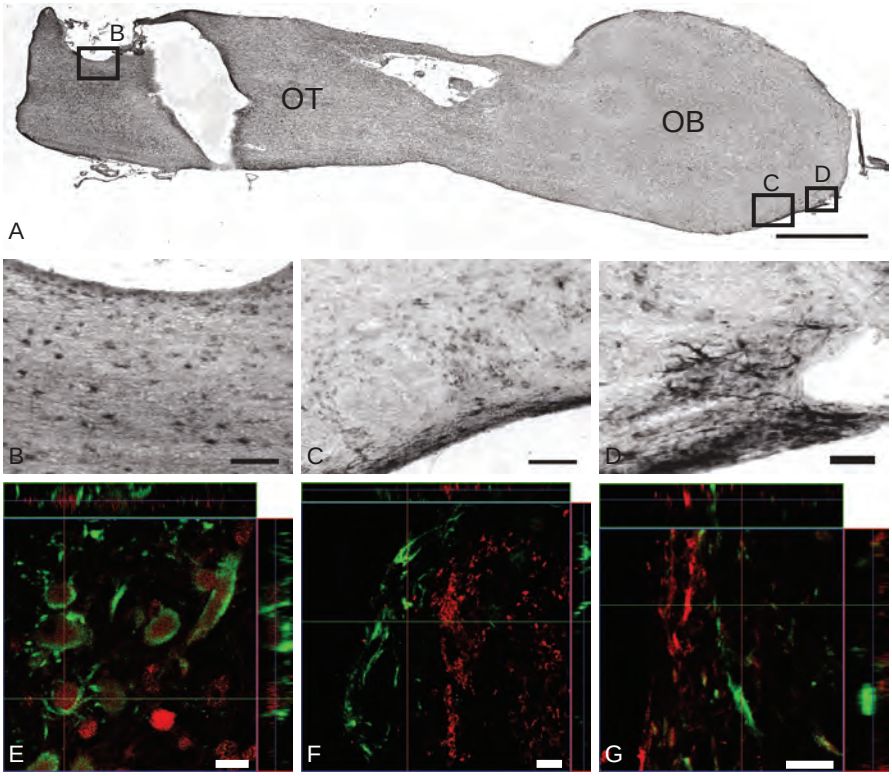


Figure 5 GFAPδ expression in the olfactory bulb and tract. (A) Overview of the olfactory bulb (OB) and part of the olfactory tract (OT) of a control donor (NBB 06-080), stained for GFAPδ. Boxed areas are enlarged in (B) for the OT and in (C) and (D) for the glomerular layer of the OB. (E) Fluorescent immunostaining of PCNA (red) and GFAPδ (green) in the glomerular layer of the OB (NBB 05-083). PCNA was expressed throughout the glomerular layer; co-expression with GFAPδ was observed in many cells. (F) Fluorescent immunostaining of βIII-tubulin (red) and GFAPδ (green) in the glomerular layer of the OB (NBB 07-014). No co-expression of these proteins was observed. (G) Fluorescent immunostaining of vimentin (red) and GFAPδ (green) in the glomerular layer of the OB (NBB 05-083). Co-expression of these proteins was only rarely observed. Scale bars indicate 2mm in (A), 100μm in B-D, 10μm in E-F.

neurospheres from several donors (data not shown). We also found GFAPδ to be expressed in newly differentiated astrocytes (Fig. 6F). This is in contrast to our findings in primary astrocyte cultures isolated from adult human brain, where it was rare to see GFAPδ staining (unpublished data).

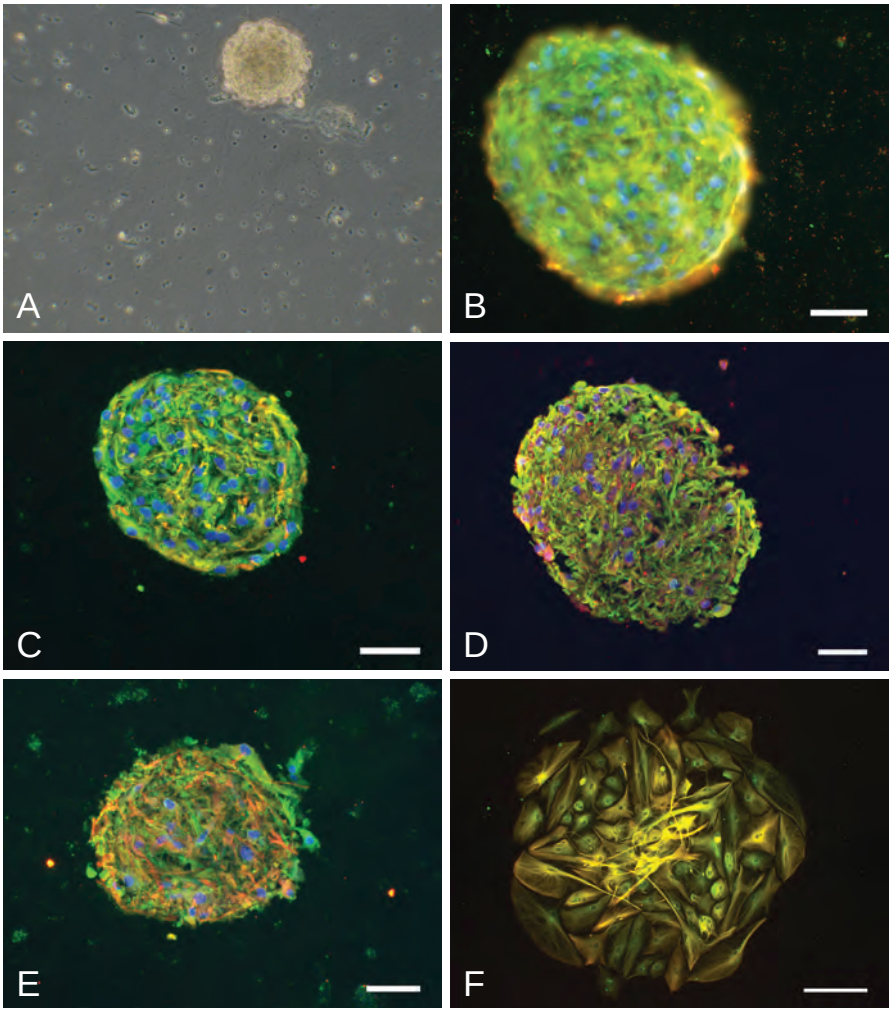


Figure 6 GFAPδ in neurosphere cultures from postmortem human brain. (A) Neurosphere from donor NBB 08-008 after 15 days in culture. (B) Neurosphere cryosection from NBB 08-076, stained for GFAPδ (green) and GFAPα (red). (C) Neurosphere cryosection from NBB 08-076, stained for GFAPδ (green) and nestin (red). (D) Neurosphere cryosection from NBB 08-078, stained for GFAPδ (green) and vimentin (red). (E) Neurosphere cryosection from NBB 08-078, stained for GFAPδ (green) and βIII-tubulin (red). (F) Newly differentiated astrocytes stained for pan-GFAP (red) and GFAPδ (green) 14 days after differentiation (NBB 08-029). Blue staining is Hoechst staining for cell nuclei; scale bars indicate 50μm in B-E, 200μm in F.

Discussion

In this study, we have shown that GFAPδ expressing cells are present throughout the aged human SVZ, RMS, OT, and OB. In the SVZ and OB, GFAPδ is expressed in a subpopulation of proliferative astrocytes that express stem cell and proliferation markers and retain BrdU longterm; and in the RMS in the glial net. We also show that GFAPδ positive cells are present in neurospheres isolated from postmortem adult SVZ. Taken together, our data provide evidence that GFAPδ expressing cells in the aged human brain are slow-cycling neurogenic astrocytes and thus NSCs.

GFAPδ expression was found along the entire length of the SVZ of the lateral ventricles in a ribbon-like pattern. The width of this ribbon and the intensity of the expression in the brain material studied were variable within cases and between cases. It is known that there is a variation in hippocampal neurogenesis, of up to 300%, between different strains of mice, implying that genetic factors influence neurogenesis considerably (Kempermann *et al.* 2006). Genetic differences could therefore explain the variability in the number of NSCs, and thus the number of GFAPδ cells. Alternatively, growth factors are very important in stem cell regulation. For instance, VEGF and EGF influence GFAP positive cells in the SVZ (Gonzalez-Perez *et al.* 2009; Mani *et al.* 2009). These and other growth factors might influence the splicing of GFAP in this region. Other than genetics and growth factors, there are many factors that influence neurogenesis (reviewed in (Zhao *et al.* 2008)), such as age, exercise, nicotine and a number of medications. However, the variability we observed in the GFAPδ ribbon could not be explained by any of these factors, which were described in the medical files of the donors.

The cyto-architecture of the human SVZ has been described before (Quinones-Hinojosa *et al.* 2006), also with respect to the GFAP positive astrocytic ribbon. In part, we observed a similar pattern for pan-GFAP expression, although we saw more variation in pan-GFAP staining patterns between donors. In the control donors we used, we observed a ribbon of GFAP positive cells directly adjacent to the hypocellular gap. For GFAPδ, we saw a ribbon of positive cells in the same location as described before (Roelofs *et al.* 2005), where fewer cells were stained for GFAPδ compared to pan-GFAP. Moreover, pan-GFAP expression could be found throughout the adjacent parenchyma, whereas GFAPδ staining is almost undetectable in these regions. Our data suggest that GFAPδ expression is restricted to the subpopulation of neurogenic astrocytes in the astrocytic ribbon of the SVZ.

We support this view by showing that the GFAPδ expressing cells in the SVZ are an immature subpopulation of astrocytes that have clear NSC-like features. These cells expressed the immature astrocyte marker vimentin, and the general astrocyte marker GFAPα (Eng *et al.* 2000). Furthermore, the GFAPδ positive

cells lacked expression of GS, a marker for fully differentiated astrocytes, and of S100B, which in the rodent is not present in NSCs, but more mature astrocytes in the SVZ (Raponi *et al.* 2007). Co-localization of GFAPδ with the proliferation markers PCNA, Mcm2 (Maiorano *et al.* 2006), and BrdU indicates that these cells are proliferative. Additionally, Sox2, a transcription factor important for the maintenance of NSCs (Graham *et al.* 2003), is co-expressed with GFAPδ. The retention of BrdU in GFAPδ positive cells months after the BrdU injection suggests that these cells are slow-cycling. For the NSC marker nestin, we found a variable pattern of double-staining with GFAPδ, with numerous cells expressing both proteins, but also cells expressing either of the IF proteins. While nestin is widely used as a marker for NSCs, previous studies have shown that expression is not restricted to precursor cells and that there is little overlap with GFAP staining in the human SVZ (Gu *et al.* 2002). We were unable to reproduce this last finding; instead we saw considerable overlap in staining in certain regions, but this might be an illustration of the variability one might observe in nestin-GFAP patterns or a difference in the antibodies that were used for GFAP. We could not find double-labeling with the proliferation markers pHH3 and Ki-67, but it must be noted that only a few cells expressing these proteins could be found, consistent with previous reports (Kukekov *et al.* 1999; Quinones-Hinojosa *et al.* 2006). This might be due to the very tight regulation of these proteins, i.e. the short half-life of Ki-67 (Mandyam *et al.* 2007), and the regulation of phosphorylation of the histone H3 protein (Juan *et al.* 1998). NSCs are slow-cycling cells (Doetsch *et al.* 1997), and therefore it is not surprising that tightly regulated proliferation markers are only sparsely observed, or even not observed at all, in the aged human neurogenic brain areas. We only rarely observed co-expression between GFAPδ and EGF-R, a marker for C-cells, and between GFAPδ and βIII-tubulin, an A-cell marker, substantiating that the GFAPδ positive astrocytes are B-cells.

In the RMS, we observed GFAPδ staining mostly in glial cells surrounding the stream of neuroblasts. The cyto-architecture of the human RMS differs from the rodent, where there is a typical GFAP positive glial tube (Peretto *et al.* 1997), which is rich in extracellular matrix components (Thomas *et al.* 1996). In the human brain, the neuroblasts in the RMS are surrounded by a glial 'net' or 'mesh' (Kam *et al.* 2009), as in the rabbit (Fasolo *et al.* 2002) and adult rhesus monkey brain (Pencea *et al.* 2001). The basic structural organisation seen in the SVZ is maintained in the RMS on the side adherent to the caudate nucleus and the ependymal structure, whereas limited SVZ components are maintained on the septal or medial side of the RMS (Kam *et al.* 2009). From work in rodents and human material, it is clear that the RMS itself still contains precursor cells (Gritti *et al.* 2002; Alonso *et al.* 2008; Mendoza-Torreblanca *et al.* 2008; Kam *et al.* 2009), but from recent work, it appears

unlikely that these are GFAP positive in human adult brain, as no PCNA+/GFAP+ cells were found (Kam *et al.* 2009), which is in line with our results for PCNA and GFAPδ in this area. Thus, in the RMS, GFAPδ is most likely only expressed in cells of the glial net surrounding the migrating neuroblasts.

In the OB, GFAPδ was found mostly in the glomerular layer, which is one of the regions where neuroblast migration terminates in rodents and where neuronal turnover takes place throughout life (Bagley *et al.* 2007). In the human brain, putative precursor cells were found within several areas of the OB, among which is the glomerular layer (Liu & Martin 2003; Bedard & Parent 2004). In our experiments in the human OB, we saw that GFAPδ expressing cells expressed PCNA, indicating local proliferation of these cells. This makes these cells excellent candidates for the putative precursor cells described before in this area.

To investigate GFAPδ in the process of neurogenesis more in depth, we established postmortem human neurosphere cultures, which we described recently (Leonard *et al.* 2009). In this *in vitro* model for NSCs, we found GFAPδ expression at mRNA and protein level. GFAPδ positive cells could be found throughout the neurospheres, co-expressing GFAPα, nestin and vimentin. As in the human adult SVZ, we observed that GFAPδ and the early neuronal marker βIII-tubulin were expressed in mostly distinct cell populations. From these results, we can conclude that GFAPδ is most likely expressed in neural precursor cells of the adult human brain, as we have found for the fetal human brain (Middeldorp *et al.* in press). We are aware of the fact that the ultimate proof is still lacking, as we cannot isolate the GFAPδ cells specifically to subject them to the neurosphere assay. For this purpose, we need to find specific cell-surface markers on GFAPδ cells, enabling us to isolate them through FACsorting. Another observation from the neurosphere culture system was that newly differentiated astrocytes highly expressed GFAPδ, as demonstrated previously (Leonard *et al.* 2009), which is not the case in primary astrocytes isolated directly from postmortem human brain. This indicates that GFAPδ is also an indicator of the differentiation state of cells.

The fact that NSCs express GFAPδ has important functional implications. Firstly, GFAPδ can destabilize the existing cytoskeleton of a cell (Nielsen & Jorgensen 2004; Roelofs *et al.* 2005; Perng *et al.* 2008), which might make a cell more flexible. This can potentially facilitate proliferation, as it is known that a cell must retract its processes, before division (Coskun *et al.* 2007). Also, an increased flexibility of the cytoskeleton is necessary for the migratory capacity of cells, and GFAP has been shown to have an effect on astrocyte motility (Lepekhin *et al.* 2001). GFAPδ could thus alter motility of migrating cells as well, by altering the properties of the cytoskeleton. This is in part supported by the morphology of GFAPδ cells, which are often elongated in a direction parallel to the ependymal lining of the SVZ.

Another aspect of GFAPδ expression in NSCs is its influence on cell signalling, as it can modulate Notch (Nielsen *et al.* 2002) and Jnk (Perng *et al.* 2008) signalling. An example of IF-mediated signalling is the regulation of the apoptosis-inducing activity of Cdk5 by nestin (Pallari & Eriksson 2006), which may be the function of nestin in NSCs. A possible function of GFAPδ in NSCs might be the modulation of Notch and/or Jnk signalling in stem cells.

In summary, we observed GFAPδ expression in quiescent NSCs in the adult human SVZ, in cells of the glial net of the RMS, in precursor cells in the OB, and in newly differentiated astrocytes *in vitro*. These results are based on co-localization studies with several markers of neurogenesis, which have been validated extensively in rodents. For humans, results have been more ambiguous, e.g. for nestin (Gu *et al.* 2002) and doublecortin (Verwer *et al.* 2007). Therefore, all results from human studies must be interpreted with caution, but we believe that with the various markers we have used, we can state that GFAPδ is most likely a marker for NSCs/B-cells at least in the SVZ. The presence of GFAPδ in some C- and A-cells and young astrocytes could represent a transitional stage between the stem cells and their differentiated progeny. Expression of GFAPδ in the RMS glial net suggests that it may also be important for the neurogenic niche. GFAPδ positive cells in the OB may represent local precursor cells, but the stem cell pool in this region of the human brain has not been studied extensively yet.

Understanding the regulation of neurogenic cells is of importance with regard to neurological disorders where replacement of neurons is necessary, such as in Alzheimer's disease, Parkinson's disease, and stroke. The fact that NSCs can be found even in the aged human brain (Leonard *et al.* 2009) gives hope that when the precise regulation of NSCs is understood, endogenous repair by these cells may be feasible.

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Supplemental data

Supplemental experimental procedures

Immunohistochemistry

For the SVZ material, 8µm-thick frontal sections of paraffin-embedded SVZ material were mounted on SuperFrost plus glass slides (Emergo, Waltham, MA, USA), deparaffinised, rehydrated, and washed with Tris-buffered saline (TBS: 0.025 M Tris, 0.14 M NaCl, pH 7.6). The sections were exposed to 20 min of heating in a steamer in citrate buffer (10 mM citric acid + 0.05% Tween-20, pH 6.0; 98°C), to provide optimal antigen retrieval. After the sections had cooled down to room temperature (RT), they were pre-incubated with TBS with 2% normal horse serum (NHS, Invitrogen-Life Technologies, Carlsbad, CA, USA), 1% bovine serum albumin (BSA) (Roche diagnostics, Mannheim, Germany), 0.1% Triton X-100, 0.05% Tween 20 to block non-specific staining, and subsequently they were incubated for 16-24 h with GFAPδ antiserum (see table 1) diluted in TBS-BSA (TBS with 1% BSA) at 4°C. The sections were then washed 3x 5 min with TBS and incubated for 1 h at RT with biotinylated goat anti-rabbit (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:400 in TBS-BSA, washed again and incubated for 1 h at RT with avidin-biotin complex (ABC; Vector Laboratories) diluted 1:800 in TBS. After the sections were washed, they were incubated for 20 min with 0.5 mg/ml 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) in TBS containing 0.04% H_2O_2. Finally, the sections were rinsed once with TBS, dehydrated in graded ethanol and xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany). Human forebrain tissue including the RMS region was cryoprotected using 30% (w/v) sucrose for 7 days before being frozen on dry ice and cut sagitally on a freezing microtome in 40mm thick sections. After extensive washing with TBS, sections were heated in a steamer for 5 min in TBS (98°C). After cooling down, sections were rinsed in TBS and treated with 0.3% H_2O_2 in TBS for 15 min at RT to block endogenous peroxidase. After blocking with 5% normal goat serum (NGS; DAKO A/S, Glostrup, Denmark) in TBS for 30 min at RT, slides were incubated 48 hours with GFAPδ antiserum diluted in 5% NGS in TBS at RT. The sections were then washed and incubated for 90 min at RT with biotinylated goat anti-rabbit 1:200 in 5% NGS in TBS, washed again and incubated for 90 min at RT with ABC complex, diluted 1:200 in TBS. After the sections were washed, they were incubated with DAB as before. Finally, sections were mounted on glass slides with 0.3% gelatine in Tris-HCl (pH 7.6), dehydrated and cover-slipped.

Paraffin-embedded OBs were cut into 20μm thick sections, which were mounted on Superfrost plus glass slides, deparaffinised, and washed with TBS. Then, the sections were exposed for 30 min to heating in a steamer in TBS (98°C). Afterwards, sections were treated similar to the immunohistochemical protocol described for the RMS. Briefly, sections were incubated with GFAPδ antiserum in 5% NGS in TBS with 0.1% Triton X-100, overnight at RT. GFAPδ was visualized using biotinylated goat anti-rabbit in 5% NGS in TBS (1:200; 60 min; RT), ABC complex (1:200; 60 min; RT) in TBS and DAB. Finally, the sections were rinsed with TBS, dehydrated, and coverslipped.

Immunofluorescence staining

For fluorescent double-staining of the SVZ and OB, sections were pre-treated similarly to the single immunohistochemical method. After cooling down, sections were washed and incubated for 16-24 hours with GFAPδ antiserum together with one of the antibodies as mentioned in table 1. The sections were then washed and incubated for 1 hr at RT with fluorescently labeled species-specific secondary antibodies (Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA) 1:400 (SVZ) or 1:200 (OB) against the various species. Cell nuclei were counterstained using Hoechst 33258, 1:1000 in TBS (BioRad, Hercules, CA, USA) or Topro-3-iodide, 1:800 in TBS (Invitrogen). Next, sections were washed, treated with Sudan Black (0.3% Sudan Black in 70% ethanol) for 7 min to quench autofluorescence, and then washed in 70% ethanol for 50 seconds. After washing the sections in TBS, they were coverslipped with Mowiol (0.1 M Tris pH 8.5, 25% glycerol, 10% w/v Mowiol 4-88 (Sigma)).

For double-labeling of two rabbit primary antibodies, the protocol was adapted, such that first one primary antibody was applied as before, followed by an incubation with Fab-anti-rabbit Cy3 antibody (Jackson, 1:800 in TBS-BSA). After washing, sections were incubated with unlabeled Fab-anti-rabbit fragments (Jackson, 1:150 in TBS-BSA) to block all binding places on the secondary antibody. Then, the staining with the second primary antibody was carried out as before. For this protocol, we always performed control stainings omitting one of the primary antibodies, to confirm specificity of the protocol.

For double-labeling with BrdU, material was obtained from two donors who had received BrdU for diagnostic purposes (Eriksson *et al.* 1998). Striatal tissue was dissected out and post-fixed in 4% paraformaldehyde for 24 hours and then transferred into 30% sucrose solution and stored at 4°C until further processing. The tissue was then frozen and 10μm cryosections were made. These sections were briefly fixed in 4% paraformaldehyde, after which they were heated as before in citrate buffer. The sections were pre-treated with 10% NGS in TBS before an

overnight antibody incubation in 3% NGS in TBS. Further staining was performed as for the SVZ material.

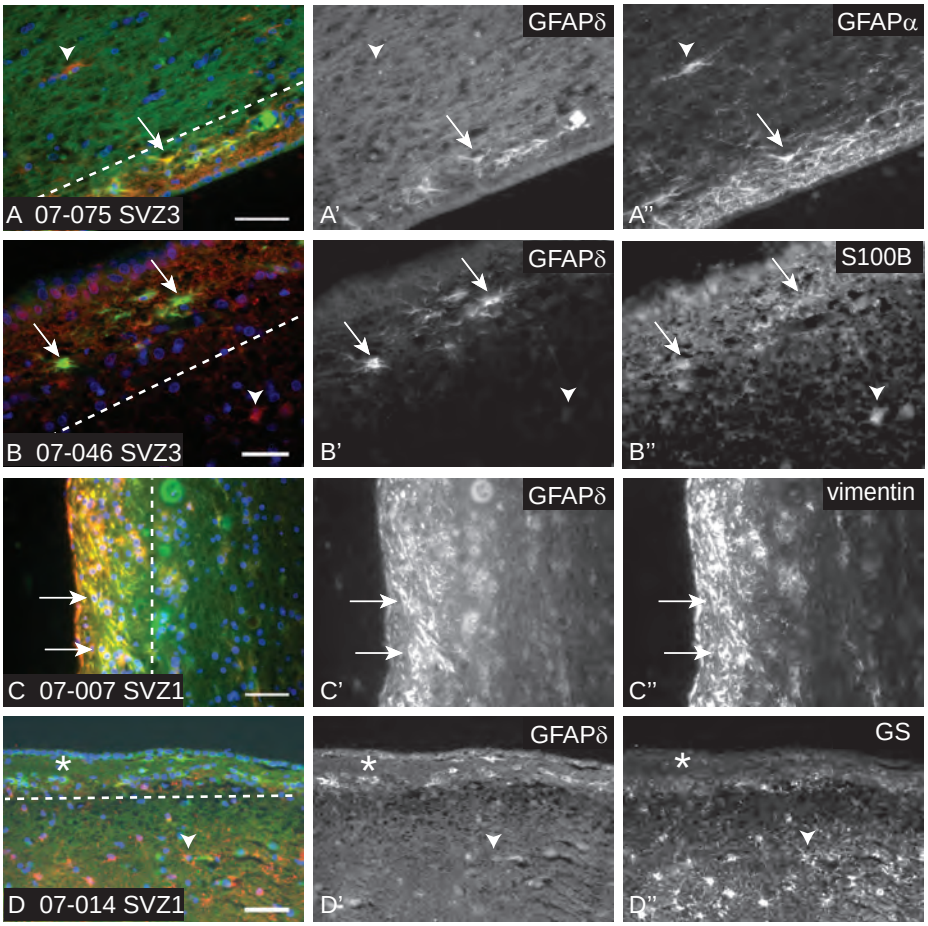
Supplemental table

Supplemental table 1 Specifications of the antibodies used

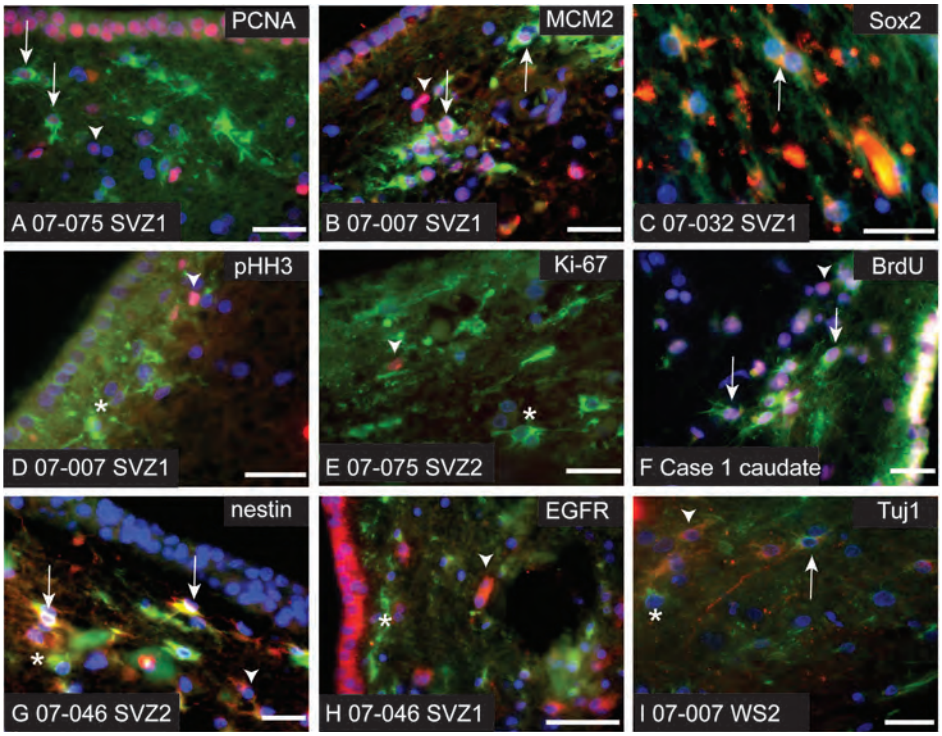
Antibody	Species	Manufacturer	Dilution
BrdU	mouse monoclonal	DAKO M0744	1:1500
C-terminal GFAP (GFAP-α)	goat polyclonal	Santa Cruz sc-6170	1:100
CD133	rabbit polyclonal	Abcam AB16518	1:100
Doublecortin	rabbit polyclonal	Abcam ab18723	1:1000
Doublecortin	goat polyclonal	Santa Cruz C-18 sc-8066	1:200
EGFR	sheep polyclonal	Upstate JBC1371207	1:100
GFAPδ	rabbit polyclonal	NIN 100501(Roelofs et al. 2005)	SVZ/C 1:500, RMS 1:1000, OB 1:100
GS	mouse monoclonal	Chemicon MAB302	1:400
Mcm2	goat polyclonal	Santa-Cruz N19 sc-9839	1:400
Nestin	mouse monoclonal	Chemicon MAB5326	1:200
NeuN	mouse monoclonal	Chemicon MAB377	1:200
Pan-GFAP	rabbit polyclonal	DAKO Z0334	1:1000
PCNA	mouse monoclonal	Santa-Cruz PC10 sc-56	SVZ/RMS 1:2000, OB 1:100
pHH3	rabbit polyclonal	Sigma H 0412	1:1000
PSA-NCAM	mouse monoclonal	Chemicon MAB5324	1:400
S100B	rabbit polyclonal	DAKO Z0311	1:600
Sox2	rabbit polyclonal	Chemicon AB5603	1:200
Vimentin	chicken polyclonal	Chemicon AB5733	SVZ/C 1:5000, OB 1:100
βIII-tubulin	mouse monoclonal	Sigma SDL.3D10	SVZ 1:400, OB 1:200

SVZ = subventricular zone, RMS = rostral migratory stream, OB = olfactory bulb, C = culture

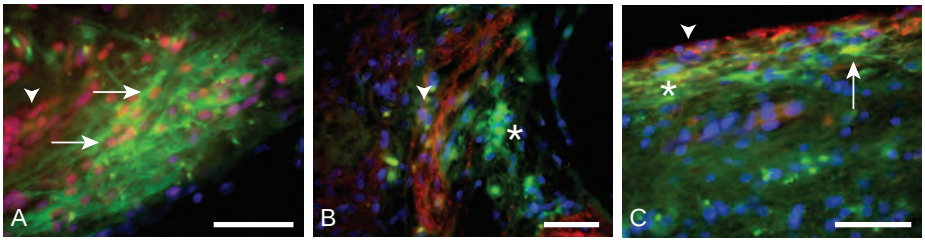
Supplemental figures



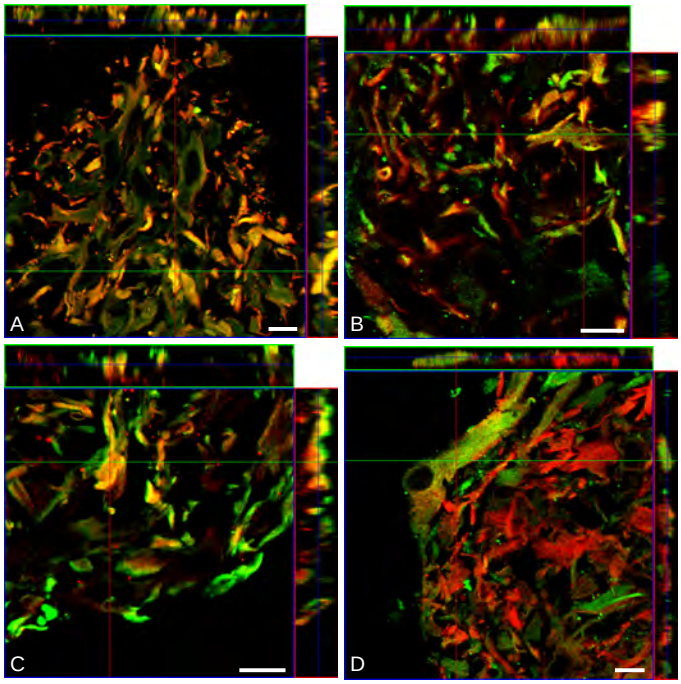
Supplemental figure S1 Fluorescent double immunostaining of GFAPδ with astrocyte markers in the SVZ. The left panel (A-D) shows the double-staining, the middle panel (A'-D') the GFAPδ staining (green), the right panel (A''-D'') represents the respective astrocyte markers, which were stained in red. A) GFAPδ is expressed in a subpopulation of GFAPα positive cells (arrow), whereas GFAPα is also expressed in the parenchyma (arrowhead). B) S100B is expressed throughout the tissue (arrowhead), co-expression with GFAPδ is found in the SVZ only (arrows). C) Vimentin expression was found mainly in the SVZ, in all GFAPδ positive cells (arrows). D) Glutamine synthetase (GS) was expressed only in the parenchyma (arrowhead), not in the SVZ. Blue staining is Hoechst staining for cell nuclei; scale bar indicates 50µm in A-C; 100µm in D; dashed line indicates border of the GFAPδ positive ribbon; individual NBB numbers of the cases are indicated in the left bottom corner.



Supplemental figure S2 Fluorescent double immunostaining of GFAPδ (green) with proliferation and stem cell markers (red) in the SVZ of different control donors. A) PCNA expression is high in the SVZ (arrows and arrowhead); most GFAPδ positive cells express PCNA (arrows). B) Most GFAPδ positive cells are Mcm2 positive (arrows). C) C) Sox2 is expressed in nuclei and cytoplasm of GFAPδ positive cells (arrow). D) pHH3 positive cells were extremely rare (arrowhead) and did not express GFAPδ (star). E) Ki-67 positive cells (arrowhead) could only be found occasionally and were not GFAPδ positive (star). F) BrdU labeling was found in many GFAPδ positive cells (arrows). G) In double immunostainings for nestin and GFAPδ, cells could be found with both proteins (arrows) and with the single proteins (arrowhead and star). H) EGF-R was not found in GFAPδ expressing cells (arrowhead and star). I) Rarely, single cells could be found expressing GFAPδ and βIII-tubulin (arrows). Blue staining is Hoechst staining for cell nuclei; scale bars indicate 25μm in all except (H), where it is 50μm; individual NBB numbers of the cases are indicated in the left bottom corner.



Supplemental figure S3 GFAPδ expression in the olfactory bulb and tract. E) Fluorescent immunostaining of PCNA (red) and GFAPδ (green) in the glomerular layer of the OB (NBB 05-083). PCNA was expressed throughout the glomerular layer (arrows and arrowhead); co-expression with GFAPδ was observed in many cells (arrows). F) Fluorescent immunostaining of βIII-tubulin (red) and GFAPδ (green) in the glomerular layer of the OB (NBB 07-014). No co-expression of these proteins was observed (arrowhead and star). G) Fluorescent immunostaining of vimentin (red) and GFAPδ (green) in the glomerular layer of the OB (NBB 05-083). Co-expression of these proteins was only rarely observed (arrow). Blue staining is Hoechst staining for cell nuclei; scale bars indicate 50μm.



Supplemental figure S4 Confocal pictures of GFAPδ in neurosphere cultures from post-mortem human brain. A) Neurosphere cryosection from NBB 08-076, stained for GFAPδ (green) and GFAPα (red). B) Neurosphere cryosection from NBB 08-076, stained for GFAPδ (green) and nestin (red). C) Neurosphere cryosection from NBB 08-076, stained for GFAPδ (green) and vimentin (red). D) Neurosphere cryosection from NBB 08-078, stained for GFAPδ (green) and βIII-tubulin (red). Scale bars indicate 10μm.