The astrocytic cytoskeleton: Unravelling the role of GFAP
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Differential role for Glial Fibrillary Acidic Protein isoforms in cell proliferation and morphology of human astrocytoma cells

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Abstract

Astrocytes are a main type of glia in the central nervous system. They are involved in neural signaling, brain homeostasis, and react to injury. Furthermore, astrocytes are adult stem cells in neurogenic niches, are able to adopt stem cell properties upon damage, and can transform into astrocytic tumors. We have previously shown that a subpopulation of neurogenic astrocytes in the subventricular zone have a specialized intermediate filament (IF) network containing a specific isoform of glial fibrillary acidic protein termed GFAPδ. Some reactive astrocytes and some astrocytic tumors also exhibit higher expression levels of GFAPδ compared to mature astrocytes in the healthy brain expressing mainly the canonical GFAPα isoform. We investigated the functional consequences of the incorporation of GFAPδ in human astrocytoma cells by studying the IF network morphology, cell migration, cell proliferation, and morphology. We observed that a high protein expression of GFAPδ results in the collapse of the whole IF network, without collapsing actin or microtubules. GFAPδ expression alters cell morphology, without affecting migration or proliferation, whereas GFAPα does increase cell proliferation. In conclusion, the morphological changes induced by GFAPδ are not directly linked to functional features of neurogenic, reactive or tumorigenic astrocyte subtypes.
Introduction

The astrocytes in the central nervous system (CNS) are multifunctional glial cells, with a diverse range of functions such as, regulation of neurotransmitter recycling, maintenance of potassium and water homeostasis, and being part of the blood brain barrier (Hewett, 2009; Wang and Bordey, 2008). In addition, astrocytes actively participate in neuronal communication via the tripartite synapse (Araque et al., 1999; Haydon, 2001; Perea and Araque, 2010). In brain disorders such as Alzheimer’s disease or stroke, astrocytes become reactive and contribute to the inflammatory response in the brain (Eddleston and Mucke, 1993; Orre et al., 2013; Sofroniew and Vinters, 2010). In specific brain areas, such as the subventricular zone (SVZ) and the hippocampal subgranular zone, a subset of astrocytes are neurogenic and act as the neural stem cells (NSCs) of the adult brain (Doetsch, 2003; Kriegstein and Alvarez-Buylla, 2009; Middeldorp and Hol, 2011). It has been hypothesized that astrocyte tumors may arise from these neurogenic astrocytes (Reya et al., 2001).

Astrocytes are classically identified by the expression of glial fibrillary acidic protein (GFAP), which is an intermediate filament (IF) protein. The IFs, together with actin filaments and microtubules form the main constituents of the cell cytoskeleton (Chang and Goldman, 2004). IFs make the cell resistant to mechanical stress, and are implicated in cell migration, autophagy, positioning of cell organelles, and cell signaling (Bandyopadhyay et al., 2010; Busch et al., 2012; Potokar et al., 2010; Sahlgren et al., 2006; Toivola et al., 2005). Intermediate filaments are linked to the rest of the cytoskeleton via linker proteins like plectin (Foisner et al., 1995; Svitkina et al., 1996), thus creating an integrated system of cytoskeleton proteins.

IFs are expressed in a highly tissue and cell type specific manner (Toivola et al., 2005). Therefore, it is thought that IFs play a pivotal role in cell type specific functions. GFAP, together with vimentin, nestin and synemin, make up the IF network in astrocytes. The combination of expression of these IFs varies greatly between astrocyte subtypes, severity of cell reactivity, and maturation stages (Izmiryan et al., 2010; Kimelberg, 2004; Lin et al., 1995; Messam et al., 2002; Middeldorp et al., 2010; Stagaard and Mollgard, 1989; Walz, 2000; Yamada et al., 1992). The best known change in IF expression occurs when astrocytes become reactive. This is accompanied by a marked upregulation of GFAP, vimentin and nestin expression. Reactive astrocytes are functionally very different from non-activated astrocytes in that they can regain their potential to proliferate, express different cytokines, secrete specific extracellular matrix components, and change their overall molecular make up (Buffo et al., 2010; Sofroniew, 2009). The functional consequences of the changes in the IF network are still elusive.
The GFAP gene gives, in addition to the canonical α isoform, rise to 9 other isoforms in humans (Blechingberg et al., 2007; Hol et al., 2003; Middeldorp and Hol, 2011; Nielsen et al., 2002; Roelofs et al., 2005). Although GFAP has always been used as a general astrocyte marker, we are only just now beginning to understand the function of GFAP. In the study presented here, we focus on GFAPα and GFAPδ. The GFAPδ protein differs from GFAPα in its C-terminal tail which is one amino acid shorter. More importantly, the last 41 amino acids

![Figure 1. GFAP isoform specific overexpression models. GFAP mRNA levels were determined in U251 cells (A, B) and in primary human astrocytes (D, E), 7 days after transduction with GFAPα or GFAPδ lentiviral constructs. The transduced GFAP isoforms gave the highest expression in both U251 (n=4) and primary human astrocytes (n=3). The overall expression levels of GFAP are higher in primary human astrocytes compared to U251 cells. Overexpression is confirmed at protein level with Western blot. In U251 cells the specific upregulation was shown with isoform specific antibodies: The GFAP c-term antibody distinctively recognizes GFAPα and the hGFAPδ antibody recognizes GFAPδ (Roelofs et al., 2005). The band in the control condition was recognized by a pan GFAP antibody and reflects the endogenous presences of mostly GFAPα. Due to less sensitivity of the C-term antibody this band is not visible when blots are stained with this antibody. (F) qPCR analysis showing GFAP isoform expression levels relative to GFAPα in untransduced U251 cells and primary human astrocytes.](image-url)
GFAP isoforms in human astrocytoma cells

differ due to alternative splicing of intron 7. GFAPδ expression is differential; only observed in subsets of astrocytes or under specific conditions. The neurogenic astrocytes, or NSCs, in the SVZ of the adult human brain express GFAPα together with high levels of GFAPδ (Roelofs et al., 2005; van den Berge et al., 2010). These cells proliferate throughout life to generate new interneurons in the olfactory bulb (Lois and Alvarez-Buylla, 1993; Sanai et al., 2004). The neurogenic astrocytes express vimentin as well as nestin, whereas mature cortical astrocytes do not. GFAPδ is also expressed in certain types of reactive gliosis and is present in some glioblastomas (Andreiuolo et al., 2009; Choi et al., 2009; Heo et al., 2012; Martinian et al., 2009). GFAPδ protein expression has been correlated to malignancy grade in glioblastomas (Choi et al., 2009; Heo et al., 2012).

The functional consequences of a high expression of GFAPδ is not completely clear, but we have found that GFAPδ, although assembly compromised on its own, is able co-assemble into an IF network with GFAPα. However, when GFAPδ protein expression becomes too high, the GFAP network collapses and condensates near the nucleus (Nielsen and Jorgensen, 2004; Roelofs et al., 2005). Stress regulated proteins like α-B-Crystallin and phosphorylated c-Jun N terminal kinase (p-JNK) (Perng et al., 2008) co-localize with the collapsed network suggesting a molecular response to the collapse. Studies from GFAP and GFAP/Vimentin knockout mice have shown that astrocytes lacking IFs have decreased cell motility and a rounder cell morphology (Lepekhin et al., 2001), although GFAP induction has been found to also decrease cell motility (Elobeid et al., 2000). In addition, the expression of GFAP has been studied in tumor cells and has been found to decrease proliferation (Rutka et al., 1994; Rutka and Smith, 1993). To further analyze the functional consequences of the different GFAP isoforms, we have created astrocytoma cell lines overexpressing GFAPα or GFAPδ and assessed the effect of the altered GFAP network on the other IF proteins and the consequences for cell morphology. Furthermore, we determined the effect on cell migration capacity and proliferation since it has been reported that GFAP affects these processes (Elobeid et al., 2000; Lepekhin et al., 2001; Rutka et al., 1994; Rutka and Smith, 1993).

Results

GFAP isoform expression in U251 cells and primary human astrocytes

Cellular models for studying GFAP isoform function were established by transducing U251 cells and primary human astrocytes with GFAP isoform expression vectors. Figure 1
shows endogenous (Ctrl bars) and exogenous GFAP isoform mRNA expression (GFAPα and GFAPδ bars) in U251 cells (Figure 1A, 1B) and in primary human astrocytes (Figure 1D and 1E); indeed we established a specific GFAP isoform overexpression compared to control cells. These data were confirmed with western blot analysis, on which a clear expression of either GFAPα or δ was observed. In the control (ctrl) condition, we only saw a band with the pan GFAP antibody, representing endogenous GFAPα (Figure 1C). The presence of other GFAP isoform transcripts was determined in untransduced U251 cells and primary human astrocytes using qPCR. The GFAP isoforms are depicted as a percentage of the canonical GFAPα expression. GFAPα and GFAPδ are the most abundant isoforms expressed, followed by GFAPκ. Other isoforms were expressed at a very low level (Figure 1F).

**GFAPδ perturbs the GFAP network in astrocytes**

To study whether cytoplasmic collapses of GFAP occur in U251 cells, as we have shown before in SW13 human adrenal carcinoma cells and U343 astrocytoma cells (Kamphuis *et al.*, 2012; Perng *et al.*, 2008), we transduced U251 astrocytoma cells and primary human astrocytes with GFAP isoforms and investigated IF network morphology by immunostainings for GFAP. A similar effect on IF morphology was obtained in primary human astrocytes (Figure 2A) and in human U251 astrocytoma cells transduced with GFAP isoforms (Figure 2B). The endogenous GFAP network was stained in the control condition, in which cells were only transduced with mCherry. The GFAP network was present throughout the cytoplasm up to close proximity of the cell periphery, which is visualized by actin staining. Expression of recombinant GFAPα in human astrocytes, or U251 cells, resulted in an incorporation of exogenous GFAPα into the endogenous IF network. In contrast, expression of GFAPδ led to a collapse of the GFAP network mostly in a perinuclear fashion (indicated with arrowheads in Figure 2A and 2B). As we have observed before that eGFP-GFAP fusion proteins can disturb the IF network as well (Perng *et al.*, 2008), we chose to use the internal ribosomal entry site (IRES) system, and identify the transfected cells by their enhanced green fluorescent protein

**Figure 2.** GFAP isoform expression in primary human astrocytes and U251 astrocytoma cells. (A) Human primary astrocytes transduced with GFAPα, GFAPδ or mCherry (control). Cells were stained with pan GFAP Dako antibody (GFAP) together with phalloidin (Actin) and Hoechst (Hst). The reporter showed which cells were transduced cells. Expression of GFAPα resulted in a dense GFAP network which was spread throughout the whole cell, similar to endogenous GFAP expression. Expression of GFAPδ showed a drastic redistribution of the GFAP network which collapsed in a perinuclear fashion. (B) Human U251 astrocytoma cells transduced with GFAPα, GFAPδ or mCherry (control) also showed a collapse of the GFAP network in GFAPδ transduced cells only. Scale bars represent 20 μm.
GFAP isoforms in human astrocytoma cells

A

B

GFAP isoforms in human astrocytoma cells
We observed that the perturbing effect of GFAPδ on the GFAP network is concentration dependent, and a gradual process as already described in studies by Nielsen and Perng (Nielsen and Jorgensen, 2004; Perng et al., 2008). This characteristic of GFAPδ is valid for both mouse and human GFAP, as we have shown in previous work (Kamphuis et al., 2012), and we observed the effect of GFAPδ on the GFAP network in multiple cell types such as primary human astrocytes as well as human astrocytoma cells (U251, U373 and U343)(Perng et al., 2008).

**A collapsed network cannot be reversed by expressing more GFAPα**

To study whether we can reverse the GFAPδ collapse of the IF network, we transduced U251 cells which were previously transduced with GFAPδ, with GFAPα to restore the α/δ ratio. Cells having both the GFAPα (EGFP) transgene as well as the GFAPδ transgene (mCherry) still showed a collapsed GFAP network when GFAPα was expressed (Figure 3). Cells transduced with EGFP show the same GFAP distribution, a collapse around the nucleus, as cells transduced with GFAPα, showing that a collapsed network cannot spread out again by increasing the amount of GFAPα.
Figure 4. GFAPδ collapses the whole IF network. (A) Primary human astrocytes transduced with GFAPα, GFAPδ or control plasmid stained for vimentin and actin or (B) nestin and actin. Astrocytes expressing ectopic GFAPα or mCherry, had a network that was spread throughout the whole cell while GFAPδ expressing cells showed a perinuclear collapse of both vimentin and nestin in cells positive for the reporter only (indicated with arrowheads). Scale bar represents 20 μm.
Vimentin and nestin co-collapse with GFAPδ, while actin and microtubules stay intact

To assess whether the GFAPδ protein expression results in a collapse of the complete cytoplasmic IF network, we studied the effect of the expression of GFAPδ on two other astrocytic IFs, vimentin and nestin. Vimentin (Figure 4A) and nestin (Figure 4B) were both expressed as a filamentous network in primary human astrocytes. Transduction with GFAPα had no effect on the distribution of the IF network. In contrast, transduction with GFAPδ, also led to a condensation of the vimentin and nestin network (see arrowheads). In the GFAPδ transduced astrocytes, most protein was collapsed around the nucleus. This is clearly visible together with the actin staining which did spread to the outer boundaries of the cell. The same effect was observed in the U251 cell line (not shown).

In order to check whether the additional expression of GFAPα or GFAPδ would lead to compensatory IF transcription, we analyzed the change in mRNA expression of vimentin,
nestin, and endogenous GFAPα and GFAPδ in U251 cells by using quantitative PCR. The mRNA expression levels of GFAPα (Figure 5A), GFAPδ (Figure 5B), and vimentin (Figure 5D) were not significantly changed due to an increase in either GFAPα or GFAPδ. However, we noticed a small but significant upregulation of nestin transcript in GFAPα transduced cells (p=0.03) (Figure 5C). Both vimentin and nestin protein were expressed in U251 cells (Figure 4), and a similar immunostaining was observed in the primary astrocytes (not shown). No clear increase in nestin immunostaining was observed. For vimentin the protein levels reflected the mRNA data in that there was no change in expression (Figure 5E).

**The cytoskeleton in astrocytes with a collapsed IF network**

IFs are interconnected with microtubules and actin (Foisner et al., 1995; Svitkina et al., 1996). To study whether a collapse in IFs will also affect the actin and microtubule network, we co-stained for actin and microtubules in GFAPα and GFAPδ transduced primary astrocytes. Cells transduced with the control vector showed normal actin and microtubule staining pattern (Figure 6). When cells had a high expression of GFAPα or GFAPδ, microtubules and actin filaments were still present throughout the whole cell. Thus, although the expression of high amounts of GFAPδ had a profound effect on the IF network the actin and microtubules did not show a collapse.

**The IF network and cell morphology**

Next we aimed at defining whether the IF network collapse results in a change in morphology since IF expression has been linked to cell morphology (Lepekhin et al., 2001). Morphological parameters of living U251 cells expressing different GFAP isoforms were determined by measuring the cell surface area and the perimeter. From these parameters we calculated the form factor, as described in the methods section. Forty cells were analyzed per experiment in five independent experiments. GFAPδ expression caused the cells to become rounder (0.56 ±0.006; mean ±SEM) in comparison to the control (0.49 ±0.005, p=0.009) (Figure 7A). Significant differences in perimeter (Figure 7B) and area (Figure 7C) between cells expressing GFAPδ (0.95 ±0.03 and 0.041 ±0.002, respectively) compared to GFAPα (1.09 ±0.03 and 0.051 ±0.002, respectively) were also found (p=0.02 and p=0.03, respectively).

**GFAP overexpression does not affect cell motility**

To check for changes in cell motility, we analyzed single cell motility and scratch wound healing speed. Single cell motility assays were performed on U251 cells expressing GFAPα,
Figure 6. The cytoskeleton in primary human astrocytes with a collapsed IF network. Primary human astrocytes transduced with GFAPα, control plasmid and GFAPδ, as indicated by the fluorescent reporter, showed that microtubules and actin filaments were not co-collapsing with the IF network. Microtubules and actin filaments were still present throughout the whole cells in GFAPδ transduced cells. Hst = Hoechst. Scale bar represents 20 μm.
GFAP isoforms in human astrocytoma cells

GFAPδ, or mCherry. Cells were seeded on PLL coated glass coverslips and thirty cells were analyzed per experiment in 3 independent experiments. There were no statistically significant differences in average velocity between U251 cells expressing GFAPδ (0.53 ± 0.09), GFAPα (0.44 ± 0.08), or control (0.50 ± 0.02) (p=0.9) (Figure 8A). Similar results were found in a wound healing assay where a monolayer of cells was scratched and the wound healing speed was measured over time (Figure 8G). Wound healing speed of U251 cells expressing GFAPα (52.9% ± 9.3), GFAPδ (54.7% ± 6.2), or the control (50 ± 7.3) was not different at 12.5h (p=0.7).

To verify these results, single cell motility assays were done on primary human astrocytes. Again we found no statistically significant difference in cell motility in cells expressing GFAPα (0.39 ± 0.03), GFAPδ (0.37 ± 0.02), and control cells (0.43 ± 0.009) (p=0.15) (Figure 8B).

GFAPδ overexpression does not affect proliferation

Since GFAPδ is highly expressed in proliferating astrocytes, neurogenic astrocytes (Roelofs et al., 2005; van den Berge et al., 2010), and astrocytoma cells (Choi et al., 2009; Heo et al., 2012), we determined the effect of GFAPδ expression on proliferation by performing a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). We observed no significant difference between proliferation of GFAPδ (153% ± 9.2), GFAPα (180% ± 19.8), and control (mCherry) (156% ± 5.3) (p=0.56) over a period of 48 hours for U251 cells (Figure 8C). The MTT assay in primary astrocytes also failed to show significant differences between proliferation of GFAPδ (146% ± 10.2), GFAPα (170% ± 18.6), and control (mCherry) (157% ± 5.4) (p=0.6) over a period of 48 hours.
differences between GFAPα (146% ± 3.8), GFAPδ (139% ± 12.5), and control (146% ± 14.7) (p=0.56) (Figure 8D). To confirm these results, proliferation was also assessed by staining for the proliferation marker Phospho histone H3 (PHH3). U251 cells stably expressing GFAP isoforms were plated, fixed 48 hours later, and stained for PHH3 (Figure 8E). We observed no significant difference (p=0.21) in the percentage of PHH3 positive cells between GFAPα (7.3%...
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± 1.1), GFAPδ (5.4% ± 0.4), and control (5.6% ±0.4). PHH3 staining in primary astrocytes did, however, show a significant difference between GFAPα (6.1% ±0.3) and GFAPδ (3.4% ± 0.2) (p=0.04) (Figure 8F). Taken together, these data show that GFAPδ expression did not alter cell proliferation or motility, while GFAPα led to a slightly higher proliferation rate compared to GFAPδ.

Discussion

Here we show that GFAPδ expression in astrocytoma cells has a profound effect on the distribution of the full IF network. This reorganization of the IF network alters the morphology of the cells, but has no effect on cell proliferation and cell motility.

The presence of a spread out IF network determines cell morphology

In reactive gliosis, the production of GFAP as well as other IF proteins are upregulated. This results in a more pronounced, GFAP-positive, IF network (Buffo et al., 2010; Sofroniew, 2009; Wilhelmsson et al., 2006) . GFAP has been linked to cell morphological changes in astrocytes in vitro, in which a correlation was observed between the level of GFAP expression and the number of cell protrusions (Elobeid et al., 2000; Lepekhin et al., 2001; Rutka et al., 1994; Rutka and Smith, 1993; Weinstein et al., 1991). In vivo however, there were no morphological changes in astrocytes in reactive astrocytes (Wilhelmsson et al., 2006). GFAP is expressed as different splice isoforms. The consequences of the presence of these isoforms in the IF network on cell morphology have not been studied before. We here show that GFAPδ-expressing cells, which show a collapsed IF network, are more rounded. This change is reminiscent to the morphological change in astrocytes devoid of IFs in vitro (Lepekhin et al., 2001; Rutka et al., 1994; Rutka and Smith, 1993; Weinstein et al., 1991).

Cell morphology in vitro is mainly determined by cell adhesion to the extracellular matrix. Integrins are the main linkers between the extracellular matrix and the cytoskeleton and, together with other proteins, they form the focal adhesions. Focal adhesions bind to actin filaments. Therefore, we expected that a change in cell morphology might go hand in hand with a redistribution of actin in cells expressing GFAPδ. However, this was not the case as we did not observe an obvious redistribution of actin. Thus the GFAPδ induced morphological changes are most likely not caused by a change in actin – focal adhesion interactions. Alternatively, the binding capacity of GFAPδ expressing cells to the extracellular matrix could be diminished by a change in IF - focal adhesion interaction through plectin.
Indeed, interactions between the IF protein vimentin, and mature focal adhesions have been shown to be essential for proper endocytosis spreading (Lynch et al., 2013). Vimentin can regulate adhesion and focal contact size under shear stress (Tsuruta and Jones, 2003) and controls cell adhesion strength through plectin and β3 integrins (Bhattacharya et al., 2009). Thus, GFAP could have a similar function in adhesion of astrocytoma cells to the ECM. IFs potentially could regulate the positioning of focal adhesions or could affect the strength of the binding of focal adhesions to the extracellular matrix (Kim et al., 2010; Lynch et al., 2013). Our observed change in cell morphology in cells with a collapsed IF network due to GFAPδ, shows that the IF network distribution per se can affect the shape of the cell.

**GFAP isoform specific functions in cell proliferation**

Proliferation has been linked to GFAP expression in several studies, although the published data are inconclusive. A GFAP knockdown has been shown to lead to an increase in proliferation in some studies (Pekny et al., 1998; Rutka et al., 1994), but not in others (Weinstein et al., 1991). On the other hand, an overexpression of human GFAPα in mice has been shown to decrease astrocyte proliferation (Cho and Messing, 2009). Since GFAPδ is expressed in cycling cells in the human brain, we expected an effect of GFAPδ on cell proliferation (Choi et al., 2009; Heo et al., 2012; van den Berge et al., 2010). Unexpectedly, we observed that not the expression of GFAPδ, but an enhanced expression of GFAPα resulted in a significant increase in proliferation in primary astrocytes. We detected a similar trend in the U251 astrocytoma cells. Our data shows that the increase in proliferation is either caused by the mere increase in GFAPα or that a collapse of the IF network prevents the stimulation of cell proliferation.

The collapse of the IF network due to GFAPδ expression is a gradual process (Nielsen and Jorgensen, 2004). The expression of GFAPδ is most likely tightly regulated as in vivo GFAPδ expressing have a spread out IF network. However, in pathological conditions with a high expression of GFAP, such as in astrocytoma and in Alexander disease astrocytes, IF collapses are observed (Eng et al., 1998; Sugita et al., 2013). This implies that also in these diseases a collapsed IF network could lead to a change in the interaction of the astrocytes with the ECM.

Mutations in GFAP are the cause of Alexander disease. This disease is characterized by Rosenthal fibers which consist of aggregated GFAP. It has been shown that the mutant GFAP proteins, lead to the collapse of the IF network and this results in a reduction in cell proliferation in primary mouse astrocytes and human cell lines (Cho and Messing, 2009;
Hagemann et al., 2006; Yoshida et al., 2007). In contrast, we here show that collapses caused by GFAPδ do not affect cell proliferation. The differences between these findings and ours could be either due to the mutation in GFAP or the difference between mouse and human GFAP.

**IF network collapse, due to GFAPδ, does not affect cell motility**

Several studies have found that a knockdown or knockout of GFAP increases cell motility (Lepekhin et al., 2001; Rutka et al., 1994; Rutka et al., 1998). However, we did not observe any differences in cell motility suggesting that a GFAPδ induced collapse of the IF network does not have the same effect on cell motility as a reduced GFAP expression or GFAP knockout. GFAP mutations, also causing collapses of the GFAP network, increased cell motility only when the mutation was in the rod domain and not in the tail domain of GFAP (Yoshida et al., 2007; Yoshida et al., 2009). GFAPα and GFAPδ have only a different tail, thus this might explain why we do not see differences in cell motility.

To summarize, we have shown that GFAPδ, has a profound effect on IF network morphology without altering astrocyte motility or proliferation. Although GFAPδ is expressed in more proliferative cell types with a higher migration potential, GFAPδ itself does not directly influence proliferation or migration when there is a collapse of the IF network. The function of GFAPδ in cells with a spread out IF network needs to be investigated further.

**Materials and Methods**

**Cell culture**

U251MG human astrocytoma cells (ECACC 89081403) were cultured in DMEM GlutaMAX/ Ham's F-10 Nutrient Mix 1:1 with 10% Fetal Bovine Serum (FBS), 10 U/ml penicillin G and 10 mg/ml streptomycin (1% P/S) (all Invitrogen, Carlsbad, CA)). Human embryonic kidney (HEK293T) cells were cultured in DMEM/Glutamax with 10% FBS, 1% P/S and 1% extra Glutamax (all Invitrogen). All cells were cultured in uncoated plastic flasks at 37°C in a humidified atmosphere, with 5% CO2.

**Isolation of primary human astrocytes**

Primary human adult astrocyte cultures were obtained from the Netherlands Brain Bank (NBB) freshly dissected post-mortem subcortical white matter from a 79-year-old female.
control donor (NBB 2010-038), with a post-mortem delay of <18 hours (h) and a cerebrospinal fluid pH of 6.30. The NBB performs brain autopsies with short post-mortem 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 I. et al., 2008). The tissue was collected in 25 ml cold Hibernate A (Invitrogen), and mechanically dissociated into small pieces. The tissue was digested with 0.2 % trypsin (Invitrogen) and 0.1% DNAseI (Invitrogen) at 37° C, while shaking for 30 minutes (min). Next, 2 ml FBS was added to the mixture and, subsequently, the cells were collected by centrifugation. The pellet was taken up in DMEM without phenol red containing 10% FBS, 2.5% Hepes, and 1% P/S (all Invitrogen), and the suspension was filtered through a 60 μm mesh screen. Then, Percoll (Amersham/GE Healthcare, Piscataway, NJ, USA) was added (half of cell suspension volume), and this mixture was centrifuged at 3220 relative centrifugal force (rcf) at 4°C for 30 min to separate cells, debris, and myelin. The second layer (glial cell containing fraction) was collected and washed with complete DMEM (containing 10% FBS, 1% P/S, 2.5% Hepes and 1% gentamycin, all Invitrogen). After centrifugation, the pellet was taken up in complete DMEM and cells were seeded in a 6-cm uncoated culture dish. Microglia will adhere to the dish and the astrocytes will be present in the medium. After 6 h at 37°C/5% CO2, the medium, containing astrocytes, was taken off, centrifuged, and the microglia depleted pellet was seeded onto Poly-L-lysine coated wells (PLL, Sigma-Aldrich, 15 μg/ml in PBS, 1 h at room temperature (RT)) in DMEM/Ham’s F12 GlutaMAX medium containing 5% FBS, 1% P/S and 0.25% Fungizone (all Invitrogen).

**Plasmid construction, transient transfection and virus production**

Expression vectors were prepared by cloning human GFAPα and human GFAPδ (Perng et al., 2006; Perng et al., 2008; Roelofs et al., 2005) cDNA sequences into the pIRES2EGFP (Clontech, Mountain View,CA, USA). For the GFAPδ constructs the EGFP sequence was replaced by mCherry.

Subsequently, to produce lentiviral vectors, the constructs were subcloned into a pRRL lenti backbone. Lentiviruses were produced as described before (Naldini et al., 1996a; Naldini et al., 1996b) with some alterations. In short, 10x106 HEK 293T cells were plated in a 15-cm culture dish and transfected with a total of 90 μg of the envelope (pMD2.G), packaging (pCMV-dR8.74) and p156RRL plasmid, containing different expression cassettes per dish, using PEI. In short, this consists of mixing the total 90 μg of DNA with PEI (67.5 ng/μl), incubate this for 15 min at RT, and adding it dropwise to the cell culture. The culture medium
was replaced 16 h after transfection and the medium containing viral particles was collected 24 h after transfection. Supernatants were ultracentrifuged at 22,000 rpm (rotor SW28, Beckman-Coulter) for 2.5 h. The resulting pellet was resuspended in Phosphate Buffered Saline (PBS) (pH 7.4), aliquoted and stored at -80 °C until further use.

To measure viral titers, a dilution series across five orders of magnitude of the viral stock solutions was made and HEK293T cells were transduced. After 2 days of incubation at 37°C, the number of transduced fluorescent cells at the different viral dilutions was counted and the viral titer was estimated in transducing units (TU) /ml.

Creating stable cell lines

U251 cells were transduced with lentiviral constructs with a multiplicity of infection of 10. Medium was refreshed after 16 h. To maintain a population of transduced cells, cells were sorted on their EGFP or mCherry expression using fluorescent activated cell sorting (FACS ARIA II, BD Bioscience, Franklin Lakes, NJ, USA). In between experiments, U251 cells were stained for GFAP to ensure that more than 70% of the cells were overexpressors. The primary human astrocytes were checked for fluorescent reporter expression before any analysis to ensure cells were overexpressing GFAP isoforms.

MTT assay

To measure cell proliferation, an MTT assay was performed. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced into a soluble blue formazan product by mitochondrial enzymes in living cells only. Therefore, the amount of formed formazan is proportional to the amount of living cells present (Mosmann, 1983). MTT assays were performed by plating cells in non-coated plastic 24-wells plates. To measure the amount of cells in the well at a specific moment, medium was replaced by 500 μl serum free medium containing 0.5 mg/ml MTT, which was incubated at 37°C for 2 h. Cells were subsequently lysed in 100% DMSO, which dissolves the purple formazan resulting in a color change of the DMSO. The amount of purple formazan, and therefore the amount of cells able to metabolize the MTT, was measured using a Varioskan Flash (Thermo scientific, USA) measuring the absorbance at 570 nm. Significance was tested with a Kruskal Wallis test with a Dunn's Multiple Comparison post hoc test on 3 independent experiments. Every measurement in the independent experiments was the average of a biological duplicate.
Chapter 2

**Phospho Histone H3 quantification**

To determine the number of actively proliferating cells, U251 cells expressing GFAP isoforms were plated on non coated coverslips and fluorescently stained for PHH3 together with the nuclear dye Hoechst (1:1000 dilution) (Invitrogen, Carlsbad, CA, USA). Subsequently, micrographs were taken and the number of PHH3 positive, dividing, nuclei were counted using ImagePro software (version 6.3) and the percentage of dividing cells was calculated by dividing the number of PHH3 positive cells by total number of Hoechst positive nuclei. Per experiment 5 fields of view were analyzed and averaged, each containing at least 50 cells. Data from separate experiments was corrected for inter-experimental variation as stated below. Significance was tested with a Kruskal Wallis test with a Dunn's post hoc test on data from 3 independent experiments.

**Migration assay**

To measure cell migration, a scratch assay was performed. U251 cells were plated in a 24-well plate (100,000 cells per well) coated with 20 μg/ml PLL at 37 °C for 1 h. The confluent cell monolayer was scratched with a P10 plastic pipet tip. Pictures were taken using an Axiovert 135M (Zeiss, Jena, Germany) with a Sony XCD-X700 camera (Sony, Tokyo, Japan) at the time points indicated in the results. The plate was marked to ensure pictures were taken at the same position at every time point. To quantify cell migration, the surface area not covered by cells was determined at different time points. The migration was calculated as the percentage of not covered surface area compared to t=0. A mean of 9 pictures was measured per condition, in at least 3 separate experiments. Significance was tested with a Kruskal Wallis test with a Dunn's post hoc test.

**Single cell motility assay**

Single cell motility assays were performed on a Zeiss Axiovert 2000 inverted microscope (Zeiss, Jena, Germany). A single cell suspension was plated on PLL-coated glass dishes with 4 compartments (CELLview, Greiner bio-one, Alphen a/d Rijn, The Netherlands) and allowed to adhere for at least 8 h. Dishes were kept on the microscope in a pre-heated and humidified incubation chamber (OKO labs, Italy) at 37 °C and 5% CO2. Pictures were taken every 10 min with an Axi Aqua camera (Q imaging, Canada). Cell motility was measured by tracking single cells throughout all frames of the sequence and measuring the average velocity in μm per min using the manual tracking plugin from Image J (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012 version
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1.46f). Per experiment at least 20 cells were analyzed per condition in at least 3 independent experiments. Data from separate experiments was corrected for inter-experimental variation as stated in the “Statistics and factor correction” section below. A Kruskal Wallis test with a Dunn’s post hoc test was performed to test for significance. For the primary human astrocytes, which were not sorted, we checked for GFP and mCherry expression to make sure that we only tracked transduced cells.

**Quantitative reverse transcriptase PCR (qPCR) analysis**

U251 cells were transduced like described before. Medium was refreshed after 16h. RNA was extracted 7 days after transduction. RNA was extracted from cells using TRIsure (Bioline, London, UK) and precipitated in isopropanol overnight (O/N). Five hundred nanograms of RNA was reverse transcribed into cDNA with a QuantiTect reverse transcription kit (Qiagen, Venlo, The Netherlands), as described before (Kamphuis et al., 2012). cDNA was diluted 1:20 before being used as a template in qPCR assays (SYBR® Green PCR Master Mix, Applied Biosystems, Life Technologies, Paisley, UK). qPCR conditions were similar as described before (Kamphuis et al., 2012) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

**Table 1.** qPCR Primer Pairs. Sequences of forward and reverse primers used for qPCR experiments are listed.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAPα endogenous</td>
<td>CCCACTCTGCTTTTGACTGAGC</td>
<td>CCTTCTCAGGCCTTAGGAGG</td>
</tr>
<tr>
<td>GFAPδ endogenous</td>
<td>GGTAAAGGTGGT-GAGTCTT</td>
<td>AGAGGCTGTGCTTGTGTC</td>
</tr>
<tr>
<td>Vimentin</td>
<td>CGTACGTCAAGCAATAT-GAAAGTG</td>
<td>TCAGAGAGGTCAACAAACCTG</td>
</tr>
<tr>
<td>Nestin</td>
<td>GATCTAACAGAGGAGGAAATCCAG</td>
<td>TCTAGTGCTCTATGGCTCTC-GT</td>
</tr>
<tr>
<td>GFAPα</td>
<td>CTTCTCAACCTGAGATTG</td>
<td>CACGGTCTCTACCCAGGATG</td>
</tr>
<tr>
<td>GFAPδ</td>
<td>CCGTGCAGACCTTCTCCTC</td>
<td>CGTATTGTGAGGCTTTTGA-GA-TATC</td>
</tr>
<tr>
<td>GFAPκ</td>
<td>GTCGTACGAGCCAGGGCTG</td>
<td>AGAGGCCTGCGACTGACG</td>
</tr>
<tr>
<td>GFAPβ</td>
<td>CGGGCATCGCACCAGTGC</td>
<td>ATCTGCTCTGCGCTTCG</td>
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<td>GFAPγ</td>
<td>CTCAGAAGAGGCTGAGCCCA</td>
<td>GCCTCCCAGCCTCAGGT</td>
</tr>
<tr>
<td>GFAPζ</td>
<td>GCAGTGATCACGTCTCCTG</td>
<td>GGTCCGCAGACGACATG</td>
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<tr>
<td>GFAP ΔEx6</td>
<td>TGCGCGCCACGGATC</td>
<td>CACGCTCTTACCCAGGATG</td>
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<tr>
<td>GFAP Δ135</td>
<td>TCTCGCGCAGGCGAGGAT</td>
<td>GGGAATTGCTGCTCGCTCTG</td>
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<tr>
<td>GFAP Δ164</td>
<td>GAGGCAGCGAGTATTCCC</td>
<td>CACGCTCAGCCAGGATG</td>
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<tr>
<td>GFAP ΔEx7</td>
<td>GCCAGAGAAGGCAACAGC</td>
<td>CTCCAGCAAGAGGCTCTT</td>
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</tbody>
</table>

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and hypoxanthine phosphoribosyltransferase (HPRT) were used as reference genes to normalize gene expression. Data from 4 separate experiments was factor corrected as stated in the “Statistics and factor correction” section below and tested for significance using a Kruskal Wallis test with a Dunn’s post hoc test. Primer pairs used are listed in Table 1.

**Western blots**

Cells were washed and scraped with a cell scraper into 100 µl of cold lysis buffer consisting of a suspension buffer (0.1 M NaCl, 0.01M Tris-Hcl (pH 7.6), 0.001 M Ethylenediaminetetraacetic acid (EDTA) with 1% Triton-x100) and protease inhibitors were added (100 µg/ml phenylmethanesulfonylfluoride (PMSF)(Roche Diagnostics, Switzerland) and 0.5µg/ml Leupeptin (Roche Diagnostics, Switzerland). Cells were vortexed and incubated for 5 min on ice. Subsequently, samples were spun at 11,7 x g for 1 min. Supernatant was taken off and stored at -20°C until further use. Protein concentrations were measured using a BCA kit (Pierce, Thermo Scientific), according to manufactures descriptions. Proteins were mixed with 2X loading buffer (2X: 100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.2M dithiothreitol and bromophenol blue), heated for 5 min at 95°C, and loaded on a 7.5% SDS-PAGE reducing gel. After electrophoresis, proteins were blotted on Whatman Protran membranes (GE Healthcare) using a semi-dry Trans-Blot system (Biorad, CA USA) for 60 minutes. Blots were incubated with SuMi for 10 min before they were incubated with primary antibodies at 4°C overnight. Blots were subsequently washed 3 times in TBS-T (100 mM Tris-Hcl pH 7.4, 150 mM NaCl with 0.2% Tween-20), before secondary antibodies (IRdye 800 (1:2000) (LI-COR, NE, USA) and Dylight Cy5 (1:4000) (Jackson Immune Research, USA), diluted in SuMi, were incubated at room temperature for 1 hour. Blots were washed again 3 times in TBS-T before scanning with an Odysee scanner (LI-COR, NE, USA). GAPDH was used as a loading control.

**Immunocytochemistry**

To perform immunocytochemical staining, cells were cultured on uncoated glass coverslips, fixed with 4% Paraformaldehyde (PFA), washed in PBS and incubated in SuMi buffer (50mM Tris, 150mM NaCl, 0.25 % gelatine and 0.5% Triton X-100, pH 7.4) for 10 min. Primary antibodies were diluted in SuMi and incubated at 4 °C on a shaker O/N. Cells were washed 3 times in PBS and subsequently incubated with secondary antibodies and Hoechst 33258 (1:1000 dilution) (Invitrogen, Carlsbad, CA, USA) diluted in SuMi at RT for 1 h. The antibodies used are listed in Table 2. All secondary antibodies were from Jackson
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Immune Research (USA) and diluted 1:1400 in SuMi. Cells were washed again in PBS, before the coverslips with cells were mounted on slides with Mowiol (0.1 M Tris-HCl pH 8.5, 25% glycerol, 10% Mowiol (Calbiochem, Merck Millipore, Darmstadt, Germany)). The actin network was visualized with acti-stain Phalloidin 670 (Cytoskeleton inc, Denver, CO, USA; 1:1000 dilution). All fluorescent images were taken with a Leica SP5 confocal microscope (Leica, Germany) with a 63x objective.

Table 2. List of antibodies. Name and additional information on antibodies used for immunocytochemistry and westernblots (WB). The dilutions for both protocols are listed.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>panGFAP Dako</td>
<td>Dako</td>
<td>1:4000 (1:8000 WB)</td>
<td>Z0334</td>
</tr>
<tr>
<td>hGFAPδ</td>
<td>Manufactured in house</td>
<td>1:1000 (1:1300 WB)</td>
<td>-</td>
</tr>
<tr>
<td>(10-05-2001 Bleed)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFAP c-term</td>
<td>Santa Cruz</td>
<td>1:4000 (WB)</td>
<td>Sc-6170</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Chemicon</td>
<td>1:5000 (1:8000 WB)</td>
<td>AB5733</td>
</tr>
<tr>
<td>Nestin</td>
<td>Millipore</td>
<td>1:1500</td>
<td>Mab353</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>Sigma-Aldrich</td>
<td>1:3000</td>
<td>T-6793</td>
</tr>
<tr>
<td>Phosphohistone H3 (PHH3)</td>
<td>Sigma-Aldrich</td>
<td>1:1000</td>
<td>H0412</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Abcam</td>
<td>1:4000 (WB)</td>
<td>AB14247</td>
</tr>
</tbody>
</table>

Cell morphology measurements

Phase contrast pictures were taken from the U251 cells transduced with GFAP isoforms and mCherry (control) on a Zeiss Axiovert 2000 with an Exi Aqua camera (Q Imaging, Surrey, Canada). Cell outlines were manually drawn using Image J. Area and perimeter were measured in square pixels. Form factor was calculated as , where perfect round cells will have a form factor of 1 (Lepekhin et al., 2001; Thurston et al., 1988). Five independent experiments were performed with 40 cells analyzed per experiment. Data has been factor corrected for inter-experimental variation as stated below. A Kruskal Wallis test was performed with a Dunn's Post hoc test to test for significance.

Statistics and factor correction

Data obtained from independent experiments was corrected with a factor correction program (version 10.5 2012) (Ruijter et al., 2006) when stated. Kruskal Wallis or Mann-Whitney tests were performed to test for significance. Differences were considered significant at p<0.05. All statistical tests were performed using Graphpad Prism 5 (version 5.04) (Graphpad Software Inc., La Jolla, CA, USA).
Chapter 2

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References


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