Reintroduction of specific GFAP isoforms into the adult GFAP KO mouse subventricular zone

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Abstract

Glial fibrillary acidic protein (GFAP) is an intermediate filament (IF) protein with several isoforms. GFAPα is the canonical isoform, being the most abundantly expressed GFAP isoform in the brain. GFAPδ is an alternatively spliced isoform that differs from GFAPα only in its C-terminal tail. GFAPα and GFAPδ are proposed to have different functions. For example, GFAPδ is principally linked to neurogenesis in the human brain; while abundant GFAPα expression has ties to more pathological circumstances, such as reactive gliosis and Alexander’s disease. This study aims to dissect GFAPα and GFAPδ specific consequences within the context of the adult mouse subventricular zone (SVZ). The SVZ was chosen as a model region, as it has a high endogenous expression of GFAP and lends itself to a variety of functional readouts involving neurogenesis. Lentiviral vectors were used to deliver either GFAPα or GFAPδ into the adult GFAP knockout (−/−) mouse SVZ. GFAP−/− mice were used in order to ensure that there would be no endogenous regulation of GFAP expression. Theoretically, this method allows for the study of the individual roles of GFAPα and GFAPδ in vivo. There was an activated unfolded protein response (UPR) that was differentially elicited between GFAP isoforms and did not include regulation of all classical UPR response genes. The reintroduction of either GFAPα or GFAPδ did not affect the SVZ niche, in terms of IF expression and stem cell characteristics. This data shows no direct role of a single GFAP isoform in proliferation or cell cycle timing in the mouse SVZ.
Introduction

The intermediate filament (IF) protein glial fibrillary acidic protein (GFAP) is widely used as a general marker for astrocytes. As an IF, it is part of the cytoskeleton of astrocytes together with other IFs, microtubules, and actin filaments. GFAP has many different isoforms (Kamphuis et al., 2012; Middeldorp and Hol, 2011). GFAPα, the canonical isoform, is expressed in most, but not all, mature astrocytes and is upregulated in reactive gliosis. Another GFAP isoform found in astrocytes is GFAPδ. This protein isoform is generated from the alternative splicing of the GFAP RNA. GFAPδ transcripts lack exon 8 and 9 and have an additional part of exon 7, termed exon7+. This results in a protein which differs at the most carboxy terminal part of the IF protein called the tail (C-terminal tail). GFAPδ is unable to form an IF network by itself. Moreover, GFAPδ also influences the IF distribution of the whole IF network when expressed in high amounts (Kamphuis et al., 2012; Moeton et al., submitted). GFAPδ has been linked to neurogenesis since GFAPδ is a marker for neural stem cells in the adult human subventricular zone (SVZ)(Roelofs et al., 2005). The SVZ is the largest neurogenic niche in the adult brain (Allen, 1912). Here, B1 astrocytes give rise to neuroblasts via transit amplifying progenitor cells (Doetsch et al., 1997; Doetsch et al., 1999). This process is tightly regulated from external signals, for example cues coming from non-neurogenic B2 astrocytes, as well as internal pathways, such as Notch signalling. Notch signalling is a crucial regulator of stem cells (Kanski et al., in prep). GFAPδ has been linked to Notch signalling through its interaction with the γ-secretase complex via its ability to bind to presenilin (Nielsen et al., 2002).

In the mouse brain, GFAPδ displays a much wider expression profile – being present in virtually every cell that expresses GFAPα (Kamphuis et al., 2012; Mamber et al., 2012). Therefore, GFAPδ is not a neural stem cell marker in the mouse, as it is expressed by both neurogenic B1 and non-neurogenic B2 astrocytes in the adult mouse SVZ. With this in mind, it is possible that GFAPδ may have a slightly different function in human and mouse. That said, however, both human and mouse GFAPδ has been found to influence the IF network morphology when expressed in high amounts. Overexpression of GFAPδ redistributes the IF network leading to a collapse near the nucleus (Kamphuis et al., 2012; Nielsen and Jorgensen, 2004; Perng et al., 2008; Roelofs et al., 2005). In lower concentrations however, GFAPδ is able to incorporate into the endogenous IF network. Notably the same effects observed in GFAPδ modulation are not seen when GFAPα is modulated (Kamphuis et al., 2012; Moeton et al., submitted). IF network morphology changes, induced by mutations in GFAP or
overexpression in a wildtype mouse, influence cell proliferation, cell death, and vulnerability to cell toxicity (Cho and Messing, 2009) suggesting that the IF network composition plays a functional role in mouse astrocytes. The composition of the proteins present within the network could possibly influence the function of the IF network by possessing differential binding capacities to other proteins or by altering the cytomechanical characteristics of the IF network. It was therefore hypothesized that, due to this characteristic, distinct GFAP isoforms could influence IF network composition and thereby function.

The proper composition of an IF network has direct functional consequences for a cell. Astrocytes that lack both GFAP and vimentin, and thereby an IF network, have deficits in their endocytosing capabilities. This deficit was shown using GFAP/ vimentin knockout (KO) mice. GFAP/vimentin KO astrocytes showed a marked decrease in the endocytosis of the Notch ligand Jagged1. This finding directly translated into functional effects namely, an increase in newborn neurons and the promotion of their survival (Wilhelmsson et al., 2012). GFAP/vimentin KO mice have also been reported to have deficits in glutamate transport, vesicle transport (Potokar et al., 2010) and glia scar formation (Li et al., 2008; Pekny et al., 1999).

However, it is unclear whether the lack of GFAP, the lack of vimentin, or the complete lack of a cytoplasmic IF network underlies these effects. Studies from GFAP KO mice report even subtler effects. GFAP KO mice develop normally and have no gross morphological deficits, although effects in LTP have been observed (McCall et al., 1996; Pekny et al., 1995; Shibuki et al., 1996). In addition, GFAP KO astrocytes have a relatively normal IF network, with no nestin or vimentin compensation (McCall et al., 1996). Like GFAP/ vimentin KO astrocytes, GFAP KO astrocytes show deficits in intracellular trafficking and reduced glutamate uptake (Hughes et al., 2004). However, these deficits do not lead to the same neurogenic phenotype described in GFAP/ vimentin KO mice. Instead, GFAP KO astrocytes do not affect the proliferation and differentiation potential of neural stem cells in vitro or in vivo (Wilhelmsson et al., 2012; Mamber et al., in prep). That said however, GFAP KO astrocytes do seem to contribute to a permissive environment in vivo that promotes the survival of newborn SVZ neuroblasts (Mamber et al., in prep).

With these very subtle effects in mind, it is the goal of this current study to investigate what specific roles, if any, GFAP isoforms have in vivo. To this end, GFAPα or GFAPδ was overexpressed in the adult GFAP KO SVZ. The SVZ was used as a model brain region as it has a high expression of GFAP, under normal circumstances. In addition, the SVZ lends itself to an
array of functional readouts. The effect of reintroduction of GFAP isoforms was investigated in the context of stem cell characteristics and cell cycle timing as well as regulation of the endogenous IF network. With these experiments the role of a specific IF instead of the whole IF network can be unravelled.

Methods

Mice

All experiments were performed with the approval of the Animal Experimentation Committee of the Royal Netherlands Academy of the Arts and Sciences (KNAW) with accordance to the European Community Council directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize both the number and suffering of the animals involved in the current study. The mice used in this current study carry a mutation in exon 1 of the Gfap gene, thus preventing GFAP expression. The GFAP knockout (GFAP KO) mice are on a mixed genetic background consisting of C57Bl6/129Sv/129Ola (Pekny et al., 1995). Adult (3 month old) male mice were used for all in vivo experiments. These mice were housed in a 21.5ºC housing facility with 12 hour light/dark cycles. Food and water was provided ad libitum.

Plasmid Production

cDNA sequences encoding for mouse GFAPα and GFAPδ were cloned into a IRES2 backbone (Clontech, Mountain View, USA; Kamphuis et al., 2012). Both sequences included an internal ribosomal entry site (IRES) with a fluorescent reporter (EGFP for GFAPα and mCherry for GFAPδ) downstream from GFAP cDNA ensuring transduced cells could be visualized. The GFAP cDNA with downstream reporter were subsequently cloned into a lentiviral backbone. The Lenti-GFP construct was a kind gift from Dr. Joost Verhaagen. This construct uses the cytomegalovirus (CMV) promoter to drive GFP and is flanked by a woodchuck hepatitis posttranscriptional regulatory element (WPRE) on the 3’end. The mCherry construct was constructed from the GFP construct. GFP was cut out of the Lenti-GFP plasmid and mCherry was cut out of the pCS2+_mCherry construct (a gift from Dr. Paula van Tijn) using BsrGI and BamH1. Subsequently, the mCherry fragment was ligated into the Lenti-GFP backbone creating the new mCherry plasmid.
Lentiviral Vector Production

Lentiviral production and viral tittering were performed as described previously (Naldini et al., 1996). In short, 45 μg of three plasmids (Envelope, Packaging, and Construct) were transfected into HEK 293T cells using polyethylenimine (Hendriks et al., 2007). 48 hours after transfection, conditioned medium was ultracentrifuged at 22,000 rpm (rotor SW28, Beckman-Coulter) for 2.5 hours. The resulting pellet was resuspended in PBS (pH 7.4), aliquotted and frozen at -80 °C until further use. To determine titers, viral stocks were diluted across five orders of magnitude and put on HEK 293T cells. The number of fluorescent cells at each viral dilution was then counted. Viral titer was estimated in transducing units/ ml (TU).
Reintroduction of GFAP isoforms into the GFAP null SVZ

Viral Vector Injections

Viral vector injections were carried out as previously described (Mamber, unpublished observation). In short, mice were sedated with an i.p. injection of FFM anesthesia [Hypnorm (fentanyl/fluanisone; Janssen Pharmaceuticals, Beerse, Belgium) Dormicum (midazolam; Roche, Almere, the Netherlands): Sterile Water; 1: 0.5: 2.5; Dose 10 µl/g)]. They were fixed to a stereotact and their skull was exposed. Viral vectors were delivered into the SVZ at the following coordinates: 0.6 AP, 1.4 ML, -2.2 DV; relative to Bregma. All viral vectors were titer matched (2.08 x 10^9 TU) and injected at a rate of 0.5 µl/ min, for a final injected volume of 2 µl. All GFAP isoforms were paired with an internal control such that, GFAPα-IRES-GFP was injected into the left SVZ and mCherry was injected into the right. Likewise, GFAPδ-IRES-mCherry was injected into the left SVZ and Lenti-GFP was injected into the contralateral SVZ. After surgery, mice recovered in a 37°C chamber and received postoperative analgesia (Finadyne; AUV Groothandel BV, Cuijk, the Netherlands; 1:10 in saline; i.p.).

In Vivo Quiescence Labeling with Thymine Analogs

Quiescence labeling was performed as previously described (Mamber et al., in prep.). In short, 16 days after surgery mice (n=3 per group) were given 5 iododeoxyuridine (IdU) injections over three consecutive days (34.7mg/ml in saline; i.p. 5μl/g). 24 days later, mice were given one chlorodeoxyuridine (CldU) injection (25.7mg/ml in saline; i.p. 5μl/g) and sacrifice 2 hours later. All thymidine analogues were used at an equimolar (98 mM) concentration, at a dosage shown previously to saturate all dividing cells (Vega and Peterson, 2005).

Tissue Handling

For immunohistochemistry, mice (n=3 per group) were deeply anesthetized with pentobarbital (0.15 ml/30 g, i.p) and then transcardially perfused with phosphate buffered saline (PBS; pH 7.4) and 4% paraformaldehyde (PFA) in PBS 2 hours after the last CldU injection. Their brains were subsequently rapidly dissected, post-fixed in 4% PFA for two hours and incubated with 20% sucrose in PBS overnight. The following day the brains were snap frozen and stored at -80°C until further use. A cryostat (Leica CM3050) was used to 12 µm coronal sections. These sections were then mounted on Superfrost Plus slides (Menzel- Gläser), dried and stored at -20°C until further use.

For RNA analysis, mice (n=4 per group) were asphyxiated with CO2/O2 and rapidly decapitated 6 weeks after surgery. Their brains were macrodissected into left and right olfactory bulbs, cortices, striatums, and SVZs as previously described (Mamber, unpublished
observation). Fresh material was put directly into TRIsure (Bioline) and homogenized with an Ultra Turrax.

**RNA Isolation, cDNA Synthesis, and quantitative Real-Time PCR**

Total RNA was isolated with TRIsure according to the manufacturer’s protocol. RNA concentration was calculated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized as described before (Mamber et al., 2012). In short, 500 ng of RNA was used as a template for cDNA synthesis which was performed according to manufacturer’s protocol using a Quantitect reverse transcription kit (Qiagen). Stock cDNA samples were diluted 1:20 with fresh H2O and stored at -20°C for later use in the quantitative real-time PCR (qPCR) reaction.

qPCR was carried out as previously described, primers are described in Table 1. GeNorm (Vandesompele et al., 2002; http://medgen.ugent.be/~jvdesomp/genorm/) was used to determine the most stable combination of reference genes for each data set. All transcript data was normalized to the geomean of beta-actin (ActB), Asparaginyl-tRNA synthetase (Nars), and proteasome subunit beta 5 (Psmb5).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (Kamphuis et al., 2012; Mamber et al., in prep). See Table 2 for a full list of all primary antibodies and their respective dilutions. The secondary antibodies used in this current study are as follows: Donkey anti-Goat Cy3, Donkey anti-Goat Cy5, Donkey anti-Rabbit Alexa488, Donkey anti-Rabbit Cy3, Donkey anti-Rabbit Cy5, Donkey anti-Rat Alexa488, Donkey anti-Mouse Alexa488, Donkey anti-Mouse Cy3. All secondary antibodies were diluted to a final concentration of 1:1400.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Product Information</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Rat anti BrdU (CldU)</td>
<td>AbD Serotec; Clone BU1/75</td>
<td>1:3000</td>
</tr>
<tr>
<td>Mouse anti BrdU (IdU)</td>
<td>BD-Biosciences; 347580</td>
<td>1:500</td>
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<td>Goat anti Doublecortin</td>
<td>Santa Cruz; sc-8066</td>
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<td>Goat anti GFAP C-19</td>
<td>Santa Cruz; sc-617</td>
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</tr>
<tr>
<td>Rabbit anti GFAPδ</td>
<td>Bleeding date: 10.12.2003</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti GFP</td>
<td>Chemicon; MAB3580</td>
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<td>Rabbit anti Ki67</td>
<td>Novacastra; NC-Ki67p</td>
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<tr>
<td>Rabbit anti RFP</td>
<td>MBL; PM005</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit anti Sox2</td>
<td>Millipore; AB5603</td>
<td>1:600</td>
</tr>
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Table 2. List of primary antibodies.
The stained sections were analyzed using a Zeiss Axioplan Neofluar fluorescence microscope (Zeiss, Jena, Germany).

Results

GFAP isoforms are successfully introduced into the GFAP KO SVZ

A lentiviral vector with a vesicular stomatitis envelope (VSV-G) carrying either GFAPα-IRES-GFP or GFAPδ-IRES-Cherry constructs driven by a cytomegalovirus (CMV) promoter were injected into the SVZ of adult GFAP KO mice. A lentivirus VSV-G carrying a fluorescent protein driven by a CMV promoter was injected into the contralateral SVZ as an internal control. A CMV-mCherry construct served as a control for the GFAPα condition (Figure 1A), while a CMV-GFP construct served as a control for the GFAPα condition (Figure 1C). The viral spread seen in these experiments 6 weeks post-transduction (p.t.) was similar to that seen before (Mamber, unpublished observation), with transduction along the rostral-caudal axis of the SVZ. Most of the transduced cells were located medially along the rostral caudal axis (Figure 1C), with some transduction seen in the rostral (not shown) and caudal SVZ (Figure 1A).

After 6 weeks p.t., GFAPα was successfully reintroduced into the SVZ and striatum, as seen by the presence of Gfapα transcripts within these regions (Figure 1B). Gfapδ transcripts were also observed in the SVZ and striatum. Interestingly, Gfapδ transcripts were found in the OB (Figure 1D), indicating either that neuroblasts were hit during the initial transduction or transduced B1 astrocytes passed along the GFAPδ construct to their progeny (see Mamber et al., 2010). GFAPα (Figure 1D) and GFAPδ (Figure 1F) constructs successfully reintroduced their respective proteins into the SVZ of GFAP KO mice. mCherry and GFP controls were also successfully expressed within the SVZ (Figure 1E,G).

GFAP reintroduction has no consequences on IF transcripts

The upregulation of GFAP and other IFs is a hallmark of reactive gliosis (Sofroniew and Vinters, 2010). Therefore, the consequences of the reintroduction of GFAP isoforms in relation to the IF network was investigated. Transcript data from both the SVZ and the striatum are shown as the viral spread targeted both regions. There was no significant upregulation of vimentin (Figure 2A-B) or nestin (Figure 2C-D) in either the SVZ or the striatum in GFAPα or GFAPδ conditions. In addition, other Gfap isoforms were also investigated. As expected,
Gfapκ and Gfapδ transcripts were not present in the GFAPα condition (data not shown). Likewise, Gfapκ and Gfapα transcripts were absent from the GFAPδ condition (data not shown). Alpha B-Crystallin, a heat shock protein known to be upregulated in cell stress and found to associate with collapses of GFAPδ in astrocytes (Perng et al., 2008), was also not significantly regulated after GFAPα or GFAPδ expression (Figure 2E-F). Together these

**Figure 1.** GFAP isoforms are successfully reintroduced into the GFAP KO brain. A Lentivirus VSV-G carrying either GFAPα-IRES-GFP or GFAPδ-IRES-mCherry was injected into the adult GFAP KO SVZ. Controls (GFP or mCherry) were injected into the contralateral hemisphere. The viral spread targeted the striatum and most of the SVZ as seen by GFP and mCherry staining (A,C). Notice most of the viral transduction targeted the medial SVZ (C), though transduction can also be seen in the caudal SVZ (A). Gfapa transcripts were seen most abundantly in the SVZ (p=0.028), followed by the striatum (p=0.028; B). Gfapδ transcripts followed the same pattern in the SVZ (p=0.028), but were also present in the OB (p= 0.028; D). Both GFAPα (D,E) and GFAPδ (F,G) protein expression was confirmed to be ipsilateral and most abundant within the SVZ. All transcript data are normalized to reference genes (n=4) and analyzed with a Mann Whitney. Data are displayed as mean ± s.e.m. Abbreviations: Ctx – cortex; OB – olfactory bulb; Str – striatum; SVZ – subventricular zone. Scale bars represent 250 μm in A and C, and 20 μm in D-G.

Gfapκ and Gfapδ transcripts were not present in the GFAPα condition (data not shown). Likewise, Gfapκ and Gfapα transcripts were absent from the GFAPδ condition (data not shown). Alpha B-Crystallin, a heat shock protein known to be upregulated in cell stress and found to associate with collapses of GFAPδ in astrocytes (Perng et al., 2008), was also not significantly regulated after GFAPα or GFAPδ expression (Figure 2E-F). Together these
data indicate that the reintroduction of GFAP isoforms did not elicit reactive gliosis or stress responses associated with a collapse of the IF network.

**GFAP isoform reintroduction has no effect on the SVZ niche**

As GFAP is expressed by both neurogenic B1 and non-neurogenic B2 astrocytes, the effect of GFAPα or GFAPδ reintroduction was assessed in the SVZ 6 weeks p.t. Total proliferation, as measured by Ki67 staining, and neuroblast production was measured (Figure 3A-F). GFAPα reintroduction did not shift total proliferation or neuroblast production when compared to its internal control (Figure 3E). The same holds true for GFAPδ reintroduction (Figure 3F). As expected from these immunohistochemical data, GFAPα or GFAPδ expression also did not change the expression of neurogenesis related transcripts such as Sox2 (Figure 3G,H) and Mash1 (Figure 3I,J) in the SVZ and OB. Hes5 transcript expression was also investigated, since GFAPδ has been shown to influence the Notch pathway (Nielsen et al., 2002; Kanski et al., submitted). However, Hes5 transcript expression within the SVZ remained stable throughout all conditions (data not shown).

Although the reintroduction of GFAP isoforms did not have an apparent effect on total proliferation, it could potentially have a subtle effect on cell-cycle timing. Other IFs have been
linked to the cell-cycle. For example, CDC2 and cyclin-dependent kinase 5 (CDK5) interact directly with nestin (Sahlgren et al., 2001; Sahlgren et al., 2003). For this reason, the quiescence of B1 astrocytes was investigated. For this experiment, mice received a total of five injections over 3 days of the thymidine analog IdU. As IdU is not cell-type specific, all dividing cells (B1 astrocytes, transit amplifying progenitors, and neuroblasts) incorporated IdU during these three days. A 24 day washout period then followed, allowing IdU+ neuroblasts to migrate to the OB and IdU+ transit amplifying progenitor cells to dissipate. After 24 days, the mice

Figure 3. GFAP isoform re-expression does not affect the neurogenic niche. The reintroduction of GFAPα into the adult mouse SVZ does not change the expression profile of neurogenic markers like Ki67 and DCX (A,B). The same holds true for the introduction of GFAPδ into the SVZ (C,D). Arrows indicate Ki67+ neuroblasts. The introduction of either GFAPα (E) or GFAPδ (F) does not affect the number of Ki67+ dividing cells or DCX+ neuroblasts in the SVZ, when compared to their respective controls. Unsurprisingly, the introduction of GFAPα (G,I) or GFAPδ (H,J) does not affect the transcript expression of Sox2 and Mash1 in the SVZ and the OB. Staining quantification (E,F) is displayed as number of marker+ cells per mm of SVZ (n=3). All transcript expression data (G-J) are normalized to reference genes (n=4). All data are analyzed with a Mann Whitney and displayed as mean ± s.e.m. Scale bars represent 10 μm. Abbreviations: LV – lateral ventricle; OB – olfactory bulb; SVZ – subventricular zone.
received one injection of another thymidine analog, CldU, and were sacrificed shortly after. This paradigm resulted in IdU+ quiescent B1 astrocytes, CldU+ transit amplifying progenitor cells and neuroblasts (hollow arrow), and IdU+CldU+ recycled B1 astrocytes and their immediate progeny (filled arrow, A-D). The introduction of either GFAPα (E) or GFAPδ (F) into the GFAPKO SVZ does not have an effect on the distribution of these dividing cell populations. Staining quantification (E,F) is displayed as ratio of marker+ cells over the total number of dividing cells in the SVZ (n=3). All data are analyzed with a Mann Whitney and displayed as mean ± s.e.m. Scale bars represent 20 μm. Abbreviation: LV – lateral ventricle.

**GFAP reintroduction does not elicit an immune response but triggers the UPR**

GFAP KO animals have been shown to react differently when challenged compared to wildtype animals. For example, they elicit a more severe response to traumatic brain injury (Nawashiro et al., 1998). Moreover, astrocytes have been recently shown to actively contribute to the immune response (Orre et al., 2013). Under this logic, the immune and
unfolded protein responses were investigated in the SVZ and striatum. For both GFAPα and GFAPδ conditions, there were no differences in microglia marker ionized calcium-binding adapter molecule 1 (Iba1; Figure 5A-B) or in a pro-inflammatory cytokine linked to reactive astrogliosis, Interleukin6 (Il6; Chiang et al., 1994) transcript expression (Figure 6A-B). These findings indicate the neither the viral transduction nor the introduction of GFAP isoforms into the GFAP KO brain illicit a significant immune response 6 weeks p.t.

Unfolded protein responses (UPR) are one of the mechanisms set in motion during cell stress (Walter and Ron, 2011). To investigate whether the UPR was altered after GFAP re-expression, the expression levels of different genes implicated in the UPR were measured.
In the SVZ, there was a significant upregulation of CCAAT/-enhancer-binding protein homologous protein (Chop) in the GFAPδ condition, but not the GFAPα condition, when compared to control (Figure 5E-F). Chop is a transcription factor induced by endoplasmatic reticulum (ER) stress (Ma et al., 2002). Transcript expression of activation transcription factor 4 (Atf4), located upstream of Chop in the ER stress induced pathway (Han et al., 2013), was also investigated. Surprisingly, no significant difference in Atf4 transcript expression was found in the SVZ of the GFAPα or GFAPδ conditions. However, there was a significant upregulation of Atf4 in the GFAPα transduced striatum 6 weeks p.t. (Figure 5G-H). These data suggest that the reintroduction of GFAPα and GFAPδ differentially activate the UPR.

Discussion

Although both GFAPα and GFAPδ were successfully reintroduced into the adult GFAP KO SVZ, very little functional consequences were observed 6 weeks p.t. The reintroduction of GFAPα and GFAPδ did not affect IF transcript expression, neurogenesis, or cell-cycle timing. Interestingly however, the reintroduction of GFAPα and GFAPδ had differential effects on the UPR. There are many aspects to consider when interpreting the findings of the current study. For example, the transgene expression levels induced in this study were most probably anomalous, probably not representing physiological expression levels. Moreover as astrocytes were not specifically targeted, it is highly likely that some neurons, oligodendrocytes, and microglia were also transduced. With these considerations in mind, this current study does lend insight into species differences in GFAP, regulation by GFAP of other IF transcripts, and GFAP’s interaction with the UPR.

Species differences in GFAP

GFAP has 10 known isoforms. Of these 10, 7 have been shown to be expressed in the adult mouse brain. Here, Gfapα transcripts are the most abundant, followed by Gfapδ transcripts (Kamphuis et al., 2012). GFAPδ differs from GFAPα in its C-terminal tail. The inclusion of alternative exon 7+ results in a stunted C-terminal tail. Via its differential C-terminal tail, GFAPδ is hypothesized to exert differential effects than GFAPα. While mouse GFAPα is 90.5% homologous to human, the GFAPδ tail is only 71% conserved. This 39% difference may help explain differential data from human and mouse GFAPδ experiments.

On protein level in mouse, GFAPδ can be found in virtually all cells that also express GFAPα (Kamphuis et al., 2012; Mamber et al., 2012). This GFAPδ expression profile seen
in mouse is in stark contrast to the human situation, where GFAPδ is predominantly is a predominant neural stem cell marker in the developing and adult human brain, although can also mark hippocampal and subpial astrocytes (Martinian et al., 2009; Middeldorp et al., 2010; Roelofs et al., 2005; van den Berge et al., 2010; Kamphuis et al. 2013 under review). This expression data combined with an altered C-terminal tail between species suggests that human and mouse GFAPδ may play divergent roles.

The clearest evidence for this divergence comes from neural stem cell (NSC) data. In immortalized human NSCs (ihNSCs), the overexpression of GFAPδ leads to a decreased sphere forming capacity. This observed phenotype might be caused by the ability of human GFAPδ to inhibit Notch signaling in cells with high Notch signaling (Kanski et al., submitted). However if the same experiment is repeated using mouse GFAPδ in mouse primary NSCs, no differences in sphere forming capacity or number of spheres is seen (Mamber, unpublished observation). The overexpression of GFAPα echoes these findings with GFAPδ. The overexpression of human GFAPα in ihNSCs yields a lower sphere forming capacity; while overexpression of mouse GFAPα in primary mouse NSCs has no effect on sphere formation (Kanski et al., submitted; Mamber, unpublished observation).

In this current study, the reintroduction of GFAPα or GFAPδ did not lead to an observable neurogenic phenotype. The total amount of proliferation, the amount of neuroblasts, as well as the cell-cycle timing remained stable throughout all conditions. These data were further supported by transcript data which indicated that neurogenesis related targets such as Hes5, Pax6 (data not shown), Mash1, and Sox2 (Figure 2) did not change amongst all studied conditions. Within the GFAP KO mouse SVZ, cell-cycle timing and total proliferation also did not change with respect to the wildtype situation (Mamber et al., in prep). Therefore, we have to conclude that the presence of a certain GFAP isoform is not a driving factor for mouse adult neurogenesis.

Notably although no neurogenic phenotype was observed in the GFAP KO SVZ, there was a massive downregulation of glial cell line-derived neurotrophic factor (Gdnf) transcripts. The reintroduction of GFAPα or GFAPδ is not sufficient to rescue this effect on Gdnf (data not shown). This peculiar piece of evidence suggests that the reintroduction of a particular GFAP isoform cannot compensate for the total lack of GFAP. Thereby suggesting, that GFAP exerts its effects via a balance of all its isoforms in a proper IF network together with other IFs. However, much caution must be taken when extrapolating this data to the human situation, as all evidence thus far suggests that GFAP isoforms play distinct roles in different species.
Robustness of the IF network

Markedly, not much conclusive data has been generated by overexpressing one particular isoform of GFAP. The upregulation of GFAP in vivo is linked to pathologies such as astrogliosis (Sofroniew and Vinters, 2010) and Alexander’s disease (Messing et al., 1998). These physiological models were one reason why it was decided in this current study to reintroduce GFAPα and GFAPδ into the GFAP KO mouse brain instead of using a wildtype mouse. More evidence for distinctive roles of GFAP isoforms comes from studies that subtly shift the ratio between GFAPα and GFAPδ.

Experiments where specific GFAP isoforms were knocked down, using shRNA, showed functional differences between astrocytes with different GFAP isoform composition. Human astrocytoma cells with lower GFAPα/GFAPδ ratios showed lower cell motility and increased the expression of Lama1, a transcript giving rise to a component of the extracellular matrix protein Laminin (Moeton et al. in preparation). These effects were not seen when GFAP isoforms were overexpressed in human astrocytoma cells (Moeton et al. under review). Besides the presence of different GFAP isoforms in the IF network there is, at least for some functions, a redundancy of other IF proteins like vimentin. This became apparent by comparing the GFAP KO animals to the GFAP/ vimentin KO animals. GFAP/ vimentin KO mice show an aggravated effect on astrocyte motility. This effect is less pronounced in the GFAP KO mouse (Lepekhin et al., 2001). These data indicate that the function of IFs should be seen in the context of the whole IF network, instead of in the context of one particular IF protein.

GFAP isoforms and the UPR response

Reintroduction of GFAP isoforms activates the UPR. The UPR is a pathway involved with managing cell stress and apoptosis. Traditionally, the UPR is activated when unfolded proteins accumulate at the lumen of the ER or when the demand for protein synthesis is too great (Eizirik et al., 2013; Walter and Ron, 2011). Once triggered, PKR-like ER kinase (PERK) mediates the phosphorylation of eukaryotic initiation factor 2α (ELF2α). This results in a global reduction of transcription but a transcriptional upregulation of Atf4. ATF4, in turn, leads to the upregulation of Chop. Within this pathway, there is a negative feedback loop whereby CHOP induces Gadd34 transcription. GADD34 can then dephosphorylate, and in effect deactivate, ELF2α.

Interestingly with the GFAPδ condition, Chop but not Atf4 was upregulated in the SVZ. This finding echoes vanishing white matter leukoencephalopathy where patients show a
massive upregulation of GFAPδ, an upregulation of Chop transcripts, but no Atf4 transcript upregulation (Bugiani et al., 2011; van Kollenburg et al., 2006). These similarities may suggest that GFAPδ transduction initially activates the UPR via a PERK dependent mechanism. However after 6 weeks, the UPR is essentially deactivated. Therefore earlier time points need to be studied in order to verify a PERK dependent, Atf4 mediated UPR response. On the other hand, this data may indicate that GFAPδ transduction leads to a sustained UPR response that is mediated by PERK but not ATF4. Although ATF4 is the main activator of Chop transcription, another yet to be identified protein has been shown to also be responsible for Chop transcription. This evidence came to light using PERK KO cells. Even after overexpression of ATF4, Chop expression was unable to be fully restored (Ma et al., 2002).

GFAPα reintroduction activated the UPR pathway differently than GFAPδ reintroduction. Here, Atf4 was upregulated in the striatum while Chop expression levels remained stable. One possible reason why only Atf4, and not its downstream target, was upregulated might involve ATF4 partner proteins. ATF4 is known to bind to other proteins that can either inhibit or promote its activity (St-Arnaud and Hekmatnejad, 2011). For example, the IF protein vimentin has been found to interact with ATF4 within the nucleus; thereby inhibiting ATF4’s effects on its downstream targets (Lian et al., 2009). Although this study was performed in osteoblasts, the same mechanism may also take place in the brain. It could be possible that GFAPα, itself, is inhibiting the ATF4 mediated response. However, more research in regards to GFAPα and ATF4 interaction is needed. While the UPR is involved in apoptosis, it is unlikely that the UPR response seen in this study at 6 weeks p.t. is leading to apoptosis. In order for the UPR to lead to apoptosis, both Atf4 and Chop upregulation are needed. Together, ATF4 and CHOP induce an upregulation of protein synthesis that eventually leads to apoptosis (Han et al., 2013). Moreover CHOP and ATF4 are involved in other pathways besides apoptosis such as lipid metabolism, the mediation of oxidative stress, and the promotion of cell survival (Cojocari et al., 2013; Donnelly et al., 2013; Harding et al., 2003; Wang and Guo, 2012). This evidence, in combination with stable transcript levels of c-fos, c-jun (data not shown), and Il6, indicates that GFAPα and GFAPδ reintroduction leads to an increased UPR as a restorative mechanism (Eizirik et al., 2013; St-Arnaud and Hekmatnejad, 2011).

Interestingly, the UPR has also been linked to astrocyte differentiation and reactivity. The UPR mediates the cleavage of old astrocyte specifically induced substance (OASIS; Murakami et al., 2006), which then activates transcription factors that promote Gfap transcription (Saito et al., 2012). The reintroduction of GFAP isoforms in this current study activated a more
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restorative UPR. Thereby, this UPR activation may be tied to the OASIS branch of UPR activation. If so, then during reactivity and development GFAP, itself, may be helping to orchestrate its own transcription. However, much more research must be done on the relation of GFAP to OASIS and the UPR to make any conclusive statements.

Concluding Remarks

In conclusion, the reintroduction of GFAPα or GFAPδ into the GFAP KO SVZ did not induce major changes in regards to neurogenesis or other astrocytic IFs. Perhaps with better transgene targeting, clearer effects could be visualized. In order to accomplish this, constructs should be driven by the gfa2 promoter which has been previously shown to guide astrocyte-specific transgene expression in vivo (Brenner et al., 1994). GFAPα and GFAPδ reintroduction did trigger unique UPR responses. Whether these UPR response are specific to GFAPα or GFAPδ remains to be seen, because the level at which GFAPα and GFAPδ were re-expressed was most probably far from the endogenous in vivo situation. The most important finding from this current study is that the functional consequences of IF proteins rely not on the IF protein itself, but rather on how they influence the IF network as a whole.

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