The astrocytic cytoskeleton: Unravelling the role of GFAP
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General discussion

Martina Moeton
Intermediate filaments (IFs) are a family of more than 70 proteins, which are expressed in a cell-type and tissue-specific manner (Herrmann and Aebi, 2004; Lazarides, 1982; Toivola et al., 2005). As their expression pattern is so cell type-specific, it was hypothesized that IFs play a role in cell specialized functions. Glial fibrillary acidic protein (GFAP) is the main IF expressed in astrocytes, and this protein together with vimentin and nestin is highly upregulated in reactive astrocytes (Pekny and Lane, 2007; Buffo et al., 2008; Sofroniew, 2009; Middeldorp and Hol, 2011). The main question is: What is the function of GFAP? To unravel the role of GFAP, several labs have generated GFAP knock-out (KO) mice. These mice are viable and develop normally (Gomi et al., 1995; Pekny et al., 1995; Liedtke et al., 1996; McCall et al., 1996), and their overall brain anatomy is normal as far as their astrocyte number and localization are concerned (Pekny et al., 1995), except for some white matter abnormalities and blood brain barrier leakage in older mice (Liedtke et al., 1996). Since GFAP is upregulated during reactive gliosis, GFAP KO animals were also investigated after brain injury. After cortical injury, vimentin and nestin expression were increased in astrocytes, showing that a reactive response does not require GFAP (Pekny et al., 1995; Eliasson et al., 1999). This is in compliance with a non-altered response after brain or spinal cord injury and scrapie infection (Gomi et al., 1995; Pekny et al., 1995). However, alterations were found in neuronal communication in the cerebellum (Shibuki et al., 1996) and hippocampus (McCall et al., 1996) and in responses to cervical spinal cord injury (Nawashiro et al., 1998). In vitro experiments in cells lacking GFAP show that this protein plays a part in cell morphology, cell motility, adhesion, proliferation and laminin expression (Lepekhin et al., 2001; Menet et al., 2001; Rutka et al., 1994; Weinstein et al., 1991; Chapter 5).

Besides a KO, overexpression of GFAP was also studied. The brains of mice with a GFAP overexpression show Rosenthal fibers - accumulations of GFAP in astrocytes. These Rosenthal fibers are a hallmark of Alexander disease (AxD) (Eng et al., 1998; Messing et al., 1998). These studies led to the discovery that AxD is caused by mutations in the GFAP gene (Brenner et al., 2001). Overexpression of GFAP is linked to increased cell death, disruption of the cytoskeleton, altered cell morphology, decrease in proliferation, lower proteasome activity and increased susceptibility to H2O2 (Cho and Messing, 2009; Elobeid et al., 2000; Moeton et al. Chapter 2). The knock-out, knockdown and overexpression studies show a modulatory role for GFAP in many different cellular functions. The phenotype is often subtle and more pronounced in GFAP/vimentin double knock-out mice (Eliasson et al., 1999; Larsson et al., 2004; Li et al., 2007; Pekny and Lane, 2007; Widestrand et al., 2007).
General discussion

All this shows that, although there are many cellular functions linked to GFAP, there is still no clear astrocyte-specific function of GFAP. On top of that, many studies have not taken into account that GFAP comes in different flavours, as the RNA is alternatively spliced. Besides the canonical α isoform there are 9 additional GFAP isoforms described in different species (human, mouse and rat) (Blechingberg et al., 2007; Condorelli et al., 1999; Hol et al., 2003; Nielsen et al., 2002; Zelenika et al., 1995). In my thesis, we made a start with investigating the functional consequences of GFAP isoforms in the astrocytic cytoskeleton, and I will discuss our findings here. This discussion focuses on 1) GFAP isoforms on a subcellular level, 2) functional consequences of different GFAP isoforms and 3) GFAP as a part of the whole IF network. To conclude I will give my opinion on how to unravel the role of GFAPδ and how future studies on the function of GFAP in astrocytes should be performed.

GFAP on a subcellular level

GFAPδ causes a collapse of the network

In Chapter 3, live cell imaging experiments using green fluorescent protein (GFP)-tagged GFAP show that the collapse of the network is a dynamic process, starting with a low amount of GFAPδ incorporated into the endogenous IF network. When the expression becomes too high, the network starts to collapse. In most cells, the network shrivels into an accumulation next to the nucleus. In some cells, smaller pieces of filaments are visible throughout the cell and these actively move towards one point near the nucleus and accumulate there. Especially this last observation triggered the hypothesis that the position of the accumulated GFAP is not random. A good candidate for this would be the centrosome. IF extension, which is not polarized, is dependent on active transport along microtubules (Gyoeva and Gelfand, 1991; Ho et al., 1998; Prahlad et al., 1998). When IF networks collapse, they dissociate from proteins that link them to the cell membrane, such as plectin, and the IFs seem to be actively transported to the centrosome. Co-localization studies of centrosome markers (such as γ-tubulin) and collapsed GFAPδ networks could help to elucidate the role of microtubules in a collapse. Low amounts of vimentin expression in cells lacking cytoplasmic IFs do show co-localization with the centrosome (Trevor et al., 1995) indicating a role for microtubules in IF network formation. In a study where protein aggregation was initiated by over-expression of a membrane protein, which folds inefficiently or by proteasome inhibition, the misfolded proteins aggregated at the centrosome. The vimentin network in these cells formed a cage-like
structure around these aggregated proteins. This structure of aggregated proteins was named the aggresome and its formation is microtubule-dependent (Johnston et al., 1998). The aggregated proteins in the aggresome are ubiquitinated and accumulate when the proteasome is inhibited. When proteasome degradation fails, the aggresome is targeted for clearance by means of autophagy (Chin et al., 2008). It might be that collapsed IF networks are positioned in the aggresome in a microtubule-dependent manner, awaiting clearance by the proteasome or autophagy. In astrocytoma cells with collapsed networks this clearance is not efficient as we still observe collapsed networks in cells several weeks after transduction with GFAPδ (shown in chapter 2). Experiments in GFAPδ overexpressing cells, where the microtubule network is depolymerized by nocodazole, would help to understand the role of microtubules in IF collapses.

In cells with a normally spread-out IF network there is a perinuclear net of IF proteins from which they seem to spread out to the cell periphery. In this net there is a position near the nucleus that may be the origin of the network, which could be the centrosome. Dupin and colleagues show that IF proteins have an actin-dependent role in nuclear positioning in astrocytes (Dupin et al., 2011). Studies on vimentin collapses demonstrate that actin is necessary for a perinuclear collapse and that it is possibly the retrograde movement of the actin cortex which makes vimentin collapse around the nucleus (Hollenbeck et al., 1989). In this study the process of the collapse, initiated by disrupting the microtubule network, is ATP-dependent. Disrupting the actin network does not inhibit the thickening of the vimentin filaments in the collapse, but does alter the localization of the collapse in fibroblasts (Hollenbeck et al., 1989). This shows a possible role for actin in the localization of the collapsed IF network.

The fact that a collapse of the IF network does not cause a co-collapse of the microtubule or actin networks shows that the connection between the different cytoskeleton components is severed at the moment of collapse in the cell periphery. Moreover, the IF network does not disintegrate before collapsing, which suggests that the IF network dissociates as a filamentous structure (Yoshida et al., 2009). Whether this is due to mechanical forces or to the fact that GFAPδ binding to linker proteins, such as plectin, is less tight than GFAPα is not known. Since we see no preferential localization of GFAPδ within the network in cells in vitro overexpressing GFAPδ, it does not seem likely that there is more GFAPδ at the parts of the filaments to which they are linked, for instance, the integrins.
Live cell imaging of intermediate filament networks

GFAP assembly is compromised by small mutations in the GFAP protein (Perng et al., 2006; Yoshida et al., 2009). Headless or tailless GFAP also leads to aberrant filament formation (Chen and Liem, 1994). These studies show the importance for the different domains of the protein for proper filament assembly. Adding a tag to the GFAP protein also affects GFAP assembly. However, for live cell imaging of GFAP a fluorescent tag is essential. In our live cell imaging experiments we observed that a high expression of GFP-tagged GFAPα led to a collapse of the network, while untagged GFAPα never caused a collapse. Indeed, it has been shown earlier that GFP-tagged GFAP or vimentin can affect network formation (Ho et al., 1998). Live cell imaging studies using GFP-tagged IFs might therefore measure altered protein dynamics due to the GFP tag. GFP is a relatively large fluorescent molecule. Therefore, other genetic labeling, such as tetracysteine (C4) tags, or antibody linked fluorophores are being used for live cell imaging. All these techniques have their own advantages and disadvantages (Fernandez-Suarez and Ting, 2008). Since GFAP assembly may be hindered by single mutations we attempted to use one of the smallest fluorescent molecules as fusion proteins for our live cell imaging experiments, the C4 tag. This tag consists of cysteine repeats, which are 20 aa in size and can be stained in live cells using the FlasH/ReAsh fluorescent compounds (Griffin et al., 1998). The C4 tag has been successfully used for live cell imaging before (Griffin et al., 1998). We created fusion proteins where the tetracystein tag was positioned at the N-terminal or C-terminal tag. Unfortunately, neither of these fusion proteins were able to form filamentous networks in SW13-cells, which lack cytoplasmic IFs, showing that even these small tags are too big for GFAP to assemble properly (Moeton: unpublished observations). Since the longitudinal elongation of the filaments seemed to be hampered, we cloned the C4 tag within linker 1,2 of GFAPα to make sure the N- and C-terminal sides were untagged (Fig 1A). This fusion protein, too, was unable to form filaments (Moeton: unpublished observations). A depiction of the different fusion proteins tested for proper self assembly are shown in Figure 1B. Neither of these constructs was able to form proper filaments in SW13-cells. Moreover, C4 tags need to be stained to become fluorescent. Proteins synthesized after the staining will therefore not be fluorescent. When C4 tags are used for Fluorescent Recovery After Photobleaching (FRAP) studies, the fluorescent recovery measured can only be GFAP molecules which were present at the time of staining. Proteins synthesized after the staining will not be fluorescent and can therefore not contribute to the FRAP analysis, but could be incorporated in the filaments after bleaching. Since the C4 tag did not seem to be a suitable
alternative we used the GFP fusion proteins for our studies. We used the N-terminally tagged GFP constructs, as GFAPα and GFAPδ only differ in their C-terminal tail, and we made sure we imaged the cells when expression levels were relatively low. We are aware that the dynamic properties of GFAP tagged with GFP are different from untagged GFAP, but we are focusing on differences between GFAPα and GFAPδ, which are tagged the same way. We are therefore confident that the difference in dynamics we found is due to differences in the GFAP protein isoforms. All in all, live cell imaging of IFs will need to be done by spiking the endogenous IF network with tagged IF proteins. The presence of both tagged and untagged IF proteins means the overall amount of tagged IF proteins will remain low enough for network formation instead of network collapse (Dupin et al., 2011; Yoon et al., 2001, 1998).

**Dynamic differences and phosphorylation**

We have shown in chapter 3 that the dynamic exchange of GFAPδ is slower than GFAPα. One of the ways in which IF protein exchange can be regulated is by phosphorylation. Phosphorylation residues at the N-terminal head domain affect filament assembly (Ralton et al., 1994; Kawajiri et al., 2003). Antibodies against phosphorylated residues, such as Thr7, Ser8 and Ser13, have made it possible to stain for phosphorylated GFAP. These residues are phosphorylated during mitosis (Matsuoka et al., 1992; Sekimata et al., 1996). Since GFAPα and

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**Figure 1**: GFAP protein structure and fluorescent tags. A) A GFAP dimer consists of an α helical rod domain flanked by non-helical head and tail domains. The rod domain consists of different coiled coil structures with non-coiled linkers in between. The relative size of the different domains is not shown to scale but is depicted purely for schematic purposes. B) For live cell imaging, fusion proteins of GFAPα linked to both GFP or C4 tags were constructed. GFAPα was tagged with one of the depicted tags but none of the constructs were able to form proper filaments in cells lacking cytoplasmic IFs.
GFAPδ differ in their C-terminal tail phospho-antibodies against residues at the C-terminal tail will help elucidate whether changes in phosphorylation lead to altered dynamic exchange. Such antibodies are not available yet. In addition, mutations at the C-terminal phosphorylation sites preventing phosphorylation or phospho-mimicking mutations can be tested to study the effect on GFAP dynamics.

Due to the antiparallel assembly of GFAP, the C-terminal tails of GFAPδ can be relatively close to the N-terminal heads of other IF proteins within the IF filament. Hypothetically the tail of GFAPδ could cause a steric hindrance of the phosphorylation of the head domain. In these collapsed networks, GFAPδ could hinder the phosphorylation and therefore change GFAPα dynamics. From initial experiments using the phospho-antibodies from Prof. Sekimata et al., 1996).

Figure 2: Phosphorylation of GFAP. U251 cells stably expressing GFAPδ were stained for phosphorylated GFAP. A) During cytokinesis there is phosphorylated GFAP present in the cleavage furrow at residue Thyrosine 7. B) Also the Serine 13 residue is phosphorylated in GFAPδ expressing cells during cytokinesis, similar to control cells (Sekimata et al., 1996). C) U251 cells outside mitosis do not show phosphorylation of the collapsed network at these residues.
Dr. Inagaki (Matsuoka et al., 1992; Sekimata et al., 1996) we saw that cells overexpressing GFAPδ are still able to phosphorylate GFAP at residues Thy7 (Fig 2A) and Ser13 (Fig 2B) during cytokinesis. Although these cells have low endogenous GFAP expression and the stained GFAP is mostly the exogenous isoform, we cannot fully exclude that the endogenous phosphorylated GFAP is stained in these cells. We can conclude, however, that the presence of GFAPδ in the network does not disable phosphorylation at the N-terminus of other GFAP proteins in the network. In collapsed networks, cells that were not in mitosis did not have visible staining (Fig 2C). Overall we did not observe overt differences in staining between GFAPα and GFAPδ expressing cells on phosphorylation during cytokinesis. However, it should be taken into account that only a few cells were in cytokinesis at the time of fixation and therefore only few cells contained phosphorylated GFAP at these residues. More elaborate studies will require cell synchronization before fixation of the cells.

Network insolubility: a cause or consequence of a collapse?

To date, it is still unclear what mechanism initiates the collapse of the network. Nielsen and colleagues described that the tail of GFAPδ binds to the coil2B of the rod domain and that this prevents GFAPδ to form monomers (Nielsen and Jorgensen, 2004). However, GFAPδ is capable of forming a network with other IF proteins and when expressed in low amounts incorporates into a spread-out network (Chapter 3). GFAPδ continues to be incorporated into the network that eventually leads to a collapse. We observed that the dynamic exchange is lower for GFAPδ than for GFAPα and that both exchange rates decrease when the network has collapsed. This suggests that GFAPδ is preferably located in the network or that GFAPδ alters the network so that there is overall less exchange with the soluble pool. We show in chapter 3 that expression of GFAPδ causes the ratio of soluble and insoluble GFAP to decrease. This suggests that the network contains proteins which are not likely to become soluble again. This would have to be reflected in a bigger immobile fraction of the FRAP studies. We did indeed observe a higher immobile fraction in collapsed networks (chapter 3). The fact that an exchange is still possible in a collapsed network shows that a new equilibrium of soluble and insoluble IF proteins is formed after the collapse of the network. However, a collapse could not be reversed by expressing more GFAPα (chapter 2), which shows that the collapsed network affects the dynamics of the GFAPα isoform as well. GFAPδ insolubility might therefore be the cause of the changes in solubility of the whole network. From our experiments we cannot conclude if the insolubility causes the collapse. Experiments in networks containing GFAPα or GFAPδ, where the dynamics of vimentin are measured, will help elucidate the effect of GFAP
isoforms on the whole IF network. We have summarized the changes in network dynamics in different networks in Figure 3.

**Figure 3:** Dynamics of GFAP isoforms in filamentous networks and collapsed networks. The dynamic differences between GFAP isoforms are schematically depicted. There is a constant exchange between the soluble pool and the filamentous network. This exchange is slower for GFAPδ. When there is too much GFAPδ expression the network collapses and this affects the exchange of both GFAPα and GFAPδ. This results in a lower soluble pool when the GFAP network is collapsed.

**Effect of GFAP on a functional level**

**Cell morphology**

Cell morphology is one feature which consistently changed whenever we altered the IF network (Chapter 2 and 5). In cells with a collapsed network we observed that cells have a smaller perimeter and are rounder compared to cells with spread-out IF networks. The same effect on cell morphology is seen in astrocytes devoid of cytoplasmic IFs (Lepekhin et al., 2001; Weinstein et al., 1991). In the case of a collapse, a drastic reorganization of the IF network ensues, and, as in cells with no IFs, no spread-out network is present. Since both conditions show a change in morphology it is likely that the role of IFs in cell morphology is a structural one, where the IF network holds together the overall shape of the cell. Although there is no collapse of the actin and microtubule network, there could be subtle changes in the distributions of these networks, which are responsible for the changes in cell morphology. It is unlikely that in the case of a collapsed IF network the morphology is changed through indirect pathways. Microarray analysis on cells with collapsed networks did not yield differences in
expression levels of actin or tubulin (Stassen et al. unpublished data).

Changing the IF network without collapsing it also caused differences in cell morphology. This reveals another possible role for IFs in cell morphology, namely through integrins. Integrins are the bridge between the cytoskeleton and the extracellular matrix (ECM) (Hynes, 2002). Integrins have roles in cell signaling, as they bind the cytoskeleton to the ECM, and therefore also in cell adhesion. We found that a pan-GFAP knockdown upregulated laminin binding integrin, such as α2 and α7 (Chapter 5), in human astrocytoma cells. There was also a marked change in morphology and adhesion in these cells when cultured on laminin.

We showed that altering GFAP networks can also affect cell morphology indirectly, via cell adhesion, and probably through integrin expression. Previous studies showed that knockdown of pan-GFAP lowered cell adhesion (Rutka et al., 1994) on non-specific substrate (plastic). This is in contrast to what we see in our experiments on poly-L-lysine (PLL). The laminin-dependent differences we observed are not present in cells which still have GFAPδ expression. Moreover, the pan-GFAP knockdown cells still have a spread-out vimentin network, showing that it is GFAP which is able to influence cell morphology. An overview of the functional consequences after altering GFAP isoform expression is listed in Table 1.

Regulation of integrins, laminin and plectin

We find a specific role for cells with an altered GFAPδ:GFAPα ratio in the upregulation of laminin and a downregulation of plectin. Pan-GFAP knockdown and GFAPα knockdown in astrocytomas both show that changing the IF network regulates integrin expression. How then does GFAP affect the expression levels of these genes?

A study by Rutka and colleagues showed that knockdown of pan-GFAP in U251MG

<table>
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<tr>
<th>Cells</th>
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<th>Morphology vs. control</th>
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<td>U251</td>
<td>GFAPα overexpression</td>
<td>Network</td>
<td>Not altered</td>
<td>-</td>
<td>No change</td>
<td>Lama1, ITGA7*</td>
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<td></td>
<td>GFAPδ overexpression</td>
<td>Collapsed network</td>
<td>Not altered</td>
<td>-</td>
<td>Smaller and rounder cells</td>
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<td>Pan GFAP KD</td>
<td>Network with less GFAP</td>
<td>Slightly reduced</td>
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<td>ITGA7, ITGA6</td>
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<td></td>
<td>GFAPα KD</td>
<td>Network containing GFAPδ</td>
<td>Reduced</td>
<td>Decreased</td>
<td>No change</td>
<td>Lama1, ITGB1, Plectin</td>
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ITGA = α integrin; lama = laminin; ITGB = β integrin; KD = knockdown; *Stassen et al. unpublished results

Table 1: Overview of overexpression and knockdown studies and their functional effects
cells led to upregulation of integrins β1 and β3 (Rutka et al., 1999). GFAP and vimentin play a role in vesicle transport in astrocytes (Potokar et al., 2010) and could thereby influence integrin trafficking to the membrane. To investigate the role of GFAP in integrin expression and localization, studies are required into the number of focal adhesions present on cells with different GFAP isoform expression. Studies in vimentin show that phosphorylation of vimentin plays a crucial role in trafficking of β1 integrin in fibroblasts (Ivaska et al., 2005). Phosphorylation of the N-terminus of vimentin by protein kinase C (PKC) was necessary for the fusion of integrin containing vesicle with the plasma membrane. GFAP could have a similar role in integrin trafficking, and a transcriptional feedback mechanism could be initiated when integrins do not reach the cell membrane. These data, together with our studies show that GFAP expression is able to regulate integrin expression.

Another possible way in which GFAP could influence gene expression is through modulating chromatin organization through the nuclear IFs and the nuclear lamina. The cytoplasmic IFs are coupled to the nuclear lamina through linker proteins such as plectin and IF-associated proteins such as nesprin (Wilhelmsen et al., 2005; Worman and Foisner, 2010). The cytoplasmic IFs could thereby influence the structure of the nuclear lamina which regulate gene expression (Collas et al., 2014).

To conclude: We have seen that changing the IF network has an effect on integrin expression. This could possibly alter integrin signaling, which could change gene expression profiles. Although our results are still inconclusive we do see that the expression of multiple genes which are part of the connection between the IF network and the ECM, is altered when the GFAP ratios are changed. This hints at a regulation of the ECM to IF network connection when GFAPδ is expressed.

**Cell motility**

The cytoskeleton is a major player in cell movement. Extension of filopodia during cell migration is mostly mediated by actin filaments. The IF network is integrated with the actin and microtubule network. GFAP has been linked to cell migration in several studies (Rutka and Smith, 1993; Rutka et al., 1994; Elobeid et al., 2000; Lepekhin et al., 2001). In our studies we see that altering the endogenous GFAP network composition hampers cell motility, but collapsing the whole IF network does not affect cell migration. Cell migration studies from GFAP knock out astrocytes also showed less cell migration in GFAP KO cells. It is therefore surprising that we did not find effects on cell migration, when cells have a collapsed IF
network. These results would suggest that the role of IF in cell migration is not dependent on the morphology and localization of the network, but is based on the expression levels of GFAP.

Vimentin has been studied in the context of astrocyte migration and is shown to be important for nuclear positioning in migrating astrocytes (Dupin et al., 2011). Depletion of this major IF in astrocytes altered nuclear positioning and decreased nuclear velocity during migration. The organization of the IF network near the nucleus is dependent on actin and on the nuclear envelope IF Lamin B1 (Dupin et al., 2011). During migration the vimentin protein binds to microtubules via adenomatous polyposis coli (APC), a protein that binds to the ends of microtubules (Sakamoto et al., 2013). If the role of GFAP in cell motility is comparable to the role described for vimentin, we would expect to find differences in cell motility when there is a collapse of the network. In the GFAPδ overexpressing cells, vimentin is also collapsed and still we see no difference in cell motility. This would indicate that there is no structural role for the IF network in cell motility.

Where a collapse of the network did not alter cell motility we did see reduction in cell motility when we changed the endogenous GFAPδ:GFAPα ratio in favor of GFAPδ. Although we observed reduced cell motility when cells were cultured on a non-ligand specific PLL coating, motility was reduced even further on laminin coating. These cells, with a shifted GFAPδ:GFAPα ratio, also have a decreased expression of several laminin-binding integrins and showed a reduced adhesion to laminin substrates. These results show a possible role for IF in cell motility via integrins. We have already discussed how GFAP could potentially influence integrin expression (‘Regulation of integrins, laminin and plectin’). If there is a direct link between the IF network and integrin expression, cell motility could be easily regulated by the IF network. We see reduced motility in cells with a higher GFAPδ:GFAPα ratio. These cells also have decreased plectin expression and have upregulated laminin 1a (Lama1). A schematic overview of the changes we found in the GFAP knockdown studies are shown in Figure 4. It could be that a proper connection is needed between the IF network and the ECM via plectin and integrins. If the high GFAPδ:GFAPα ratio in the network disrupts this link, it could lead to a feedback mechanism regulating integrins and plectin. To compensate for the lack of proper laminin integrin signaling, the cell upregulates laminin expression. The secretion of laminin would explain why we see a reduction in motility in cells with a higher GFAPδ:GFAPα ratio on PLL. At this point this is mere speculation and warrants further investigation. Testing this hypothesis requires investigation of the protein expression levels and localization of laminin,
plectin and integrins \( \beta1 \) and \( \alpha6 \). Should a loss of plectin be the cause of the decrease in cell motility, then re-expression of plectin should rescue the phenotype.

**Studying GFAP function within the IF network**

**Altering and not collapsing**

The GFAP protein is part of a bigger network of proteins which make up the IF network in astrocytes. Together with vimentin, nestin and synemin it creates one integrated network. It is perhaps better to assess the function of the IF network in its entirety and investigate how GFAP or GFAP isoforms affect it. Studies in vimentin GFAP double KO mice show that the effects of astrocytes devoid of both IFs are different or more exaggerated than those of single knockouts (Lepekhin et al., 2001; Pekny, 2001; Wilhelmsson et al., 2004; Widestrand et al., 2007).

The overexpression studies we performed showed a collapse of the whole IF network. Although this collapse is caused by the expression of GFAP\( \delta \), the functional effects seen could be due to the collapse of the network and not to GFAP\( \delta \) per se. Only when we altered the endogenous ratio of GFAP isoform expression to shift the GFAP\( \alpha \):GFAP\( \delta \) ratio did we find functional differences, possibly attributable to GFAP\( \delta \) expression, as we used a pan-GFAP knockdown as a control. It seems pivotal to still have GFAP\( \delta \) in a network to see its influence on IF network functioning.

**Figure 4:** Summary of functional changes after altering the endogenous GFAP composition. A schematic overview of the consequences of changing GFAP isoform composition in astrocytoma cells. Cells with a knockdown of all isoforms show slightly reduced motility and upregulate the mRNA expression of several laminin binding integrins compared to the GFAP\( \alpha \) KD. These cells have an increased adhesion to laminin. The GFAP\( \alpha \) KD cells have an altered GFAP\( \alpha \):GFAP\( \delta \) ratio, which makes them express GFAP\( \delta \) within a spreadout network. These cells show significantly lower cell motility and downregulated \( \beta 1 \) integrin expression. The GFAP\( \alpha \) KD cells upregulate Lama1 expression levels.
Differences between mouse and human

GFAPδ expression is found in a number of species, including mouse and human. The human and mouse brain differ in terms of the GFAPδ expression pattern. Mamber et al. showed that, in the mouse, most GFAP-expressing astrocytes also express GFAPδ. In the mouse SVZ the GFAPδ-expression does not mark the neural stem cells like it does in the human SVZ (Mamber et al., 2012). The C-terminal tail of GFAPδ is much less conserved between mouse, rat and human than GFAPα, suggesting a divergence in sequence, and possibly in function, in the course of evolution (Boyd et al., 2012). The binding of presenilin to human GFAPδ, for instance, could be altered or absent in mouse GFAPδ due to sequence differences (Nielsen et al., 2002). These alterations could lead to functional differences. The role of GFAP in astrocytes might also be different in mouse and humans due to differences in astrocytes between these species. Human astrocyte physiology is much more complex than that of mouse, in terms of process arborization, size and amount of sub-types (Oberheim et al., 2006, 2012), making human astrocytes much more specialized. Transplanting human astrocytes into mouse brain resulted in enhanced long-term potentiation, and therefore smarter mice (Han et al., 2013). It is not known whether GFAP plays a role in these interspecies differences but it should be kept in mind when studying astrocyte function in rodents.

Cell type and model system

Expression of GFAPδ is controlled by the splicing of the GFAP mRNA. Splicing is different in different cell types. During cell differentiation changes are also seen in splicing (Chepelev and Chen, 2013). This indicates that endogenous GFAPδ expression is only present in cells with specific expression profiles that have distinct splicing patterns. If GFAPδ has a specific function in undifferentiated cells only, this function can only be studied in certain cell types. Mature astrocytes, for instance, may lack signaling proteins, which stem cells do have.

Due to the expression of GFAPδ in the neural stem cells in the human SVZ, it has been linked to an immature state of differentiation. Functional differences between GFAPα and GFAPδ could occur through binding or regulation of proteins such as 14-3-3 or presenilin. These proteins would need to be present and active in the model system used to study GFAPδ function. GFAPδ binds presenilin, which is part of the γ-secretase complex. This protein complex cleaves different signaling molecules such as amyloid precursor protein (APP) and Notch, which play a role in stem cell maintenance and differentiation (Kageyama and Ohtsuka, 1999; Wolfe, 2009; Nalivaeva and Turner, 2013). In cells with, for instance,
low Notch signaling, a role of GFAPδ in Notch signaling would be overlooked. That GFAP function might differ depending on cell type is shown in experiments in neural stem cell lines, where GFAP decreased cell proliferation, while in astrocytoma cells we found that only GFAPα slightly increases cell proliferation (personal communications with Regina Kanski and Chapter 2). It would therefore be advisable to also study GFAPδ function in stem cell models. The study of the role of GFAPδ in stem cells requires appropriate model systems. Non-adherent stem cell models would be preferable here, as IF expression has been shown to change in 2D in vitro settings (Puschmann et al., 2013). However, GFAPδ expression is also found in astrocytoma cells. The results we find in our experiments will therefore have to be linked to GFAP isoform function in tumor cells and cannot be extrapolated to other cell types.

**Outlook**

The knockdown experiments performed in this thesis show a role for IF networks with a high GFAPδ:GFAPα ratio in communication between the ECM and the IF network, which regulate cell adhesion, cell motility, and possibly laminin expression. The molecular mechanisms behind these functional effects are unclear and need to be investigated further. Proteins such as plectin and laminin, which are in the pathway from IF network to ECM, should be altered by shRNA knockdown or overexpression to assess the role of GFAPδ in cell adhesion and motility. The study of GFAPδ function requires an alteration of the network - without collapsing it - as we found no functional differences in our overexpression studies. Regulated expression or specific knockdown of GFAPδ in different cellular models will help define the role of GFAPδ.

**GFAPδ in tumor biology**

ECM deposition and integrin expression are very important for the migration and invasiveness of glioma cells (Giese and Westphal, 1996; Rooprai et al., 1999). We showed that altering the IF network affects the adhesion and migration of astrocytoma cells. Understanding the role of GFAPδ in adhesion and migration of astrocytoma cells is important for possible prevention of tumor cell invasion. To understand the role of GFAPδ in adhesion and migration the mRNA expression data on integrins, laminin and plectin first need to be confirmed on protein levels. For integrin and plectin protein expression the localization of the proteins is also of great importance. Plectin reorganization may occur, although the expression levels stay the same, which could affect IF binding to integrins. For the integrins the membrane
bound fraction of proteins is crucial, as these may transfer signals from the ECM into the cell. Protein quantifications of integrins and plectin would therefore have to be done together with localization experiments to assess whether changes in the IF network alter the position of these proteins. In Figure 5, a schematic representation shows how GFAPδ could alter ECM cytoskeleton interactions. Deciphering the role of GFAPδ in tumor cell invasiveness may offer new possibilities for interference with tumor spreading. Altering human astrocytoma cell lines or primary tumor material in their IF network composition and transplanting them back into mice would make it possible to investigate GFAPδ function in vivo. This would be an elegant way of investigating human GFAP in human cells in an in vivo environment.

Next, it would be very interesting to unravel the molecular mechanism of the way in which IF network composition alters gene expression or cell signaling. As we found that transcripts related to integrin, plectin and laminin expression were altered, signaling pathways involving these proteins should be investigated. The phosphorylation of focal adhesion kinase (FAK),

**Figure 5:** Sites where GFAPδ may influence ECM-to-nucleus communication. Altering the GFAPδ:GFAPα ratio in the IF network leads to differences in gene expression as well as to decreased cell adhesion and migration. The IF network forms a connection between the ECM (here Laminin) and the cell nucleus, through integrins, Plectin, Nesprins and Lamins in the nuclear lamina. The effect of a changed IF network could hypothetically influence IF to ECM connections and/or IF to nuclear lamina connections. Further studies are needed to elucidate what the molecular mechanisms are behind the role of GFAPδ on migration, adhesion and laminin expression.
which is involved in cell migration and proliferation, may function as a readout for integrin signaling although it is not specific to integrin signaling (Natarajan et al., 2003).

**GFAPδ in neural stem cells**

The role of GFAPδ should also be studied in human neural stem cell (NSC) models. In stem cells the influence of GFAPδ on proliferation, stem cell quiescence and differentiation should be assessed as these are stem cell characteristics (Most of these questions are addressed in Regina Kanski’s thesis). The stem cell environment might provide binding partners of GFAPδ, which influence stem cell characteristics that might be absent in astrocytes. The possible role of GFAP isoforms in presenilin or 14-3-3 binding might be particularly important in undifferentiated cells and should therefore be studied in stem cells. There is evidence from work done by Regina Kanski in our group that a feedback loop exists between GFAP and Notch activity (Kanski, 2014). The effects of altered IF network composition on notch signaling in stem cells seems a promising line of research. For neural stem cells, too, I would suggest altering the endogenous GFAP isoform levels instead of overexpression. The role we find for GFAPδ in cell motility and adhesion is a more challenging object of study in neural stem cells in vitro, as most neural stem cells start to differentiate when plated on laminin as adherent cells.

In summary, it is important to distinguish between different GFAP isoforms when studying GFAP, especially since the different isoforms have very different effects on the IF network. Altering the endogenous GFAP isoform expression levels uncovered functional differences between GFAPα and GFAPδ, while overexpression studies did not. For future studies it would therefore be advisable to avoid overexpression models, as these cause the network to collapse. Identifying specific roles of GFAP isoforms, or different IFs for that matter, will help us understand the role of the IF network in different cell functions.
References


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