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Review

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Glial fibrillary acidic protein: from intermediate filament assembly and gliosis to neurobiomarker

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Glial fibrillary acidic protein (GFAP) is an intermediate filament (IF) III protein uniquely found in astrocytes in the central nervous system (CNS), non-myelinating Schwann cells in the peripheral nervous system (PNS), and enteric glial cells. GFAP mRNA expression is regulated by several nuclear-receptor hormones, growth factors, and lipopolysaccharides (LPSs). GFAP is also subject to numerous post-translational modifications (PTMs), while GFAP mutations result in protein deposits known as Rosenthal fibers in Alexander disease. GFAP gene activation and protein induction appear to play a critical role in astroglial cell activation (astrogliosis) following CNS injuries and neurodegeneration. Emerging evidence also suggests that, following traumatic brain and spinal cord injuries and stroke, GFAP and its breakdown products are rapidly released into biofluids, making them strong candidate biomarkers for such neurological disorders.

GFAP overview and outline

Astrocytes are a type of glial cell in the CNS, a group that also includes resident and perivascular microglia, oligodendrocytes, radial glia, and Müller cells. It is estimated that astroglial cells are the most abundant cell types in the brain, providing both structural and functional support for neurons (including neurotransmitter glutamate recycling and trophic factor release). Astrocytes (astroglia) are characterized by the presence of a unique structural protein, GFAP, isolated and characterized by Dr Eng in 1969 [1]. GFAP is known to be present in non-myelinating Schwann cells in the PNS and enteric glial cells as part of the enteric nervous system (ENS) [2,3]. In this review, we first examine the structural layout of GFAP, its various splice variants, and the pathological significance of GFAP mutations. We discuss how GFAP is regulated both at the transcriptional and the post-translational levels and how such regulations might impact on GFAP's normal cytoskeletal functions and its involvement in maintaining the activated astroglial cell state (astrogliosis) following nervous system injury. We further focus on the emerging evidence for GFAP and its modified forms as a promising protein biomarker for neurotrauma and stroke. Lastly, we also discuss how GFAP was identified as a dominant autoantigen following traumatic brain injury (TBI) and its implications in terms of triggering a possible post-TBI and sustained autoimmune response toward the nervous system.

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CNS–PNS–ENS specificity

GFAP is highly expressed in the CNS (Figure 1) [Su, A., ed. (2012) *Dataset: GeneAtlas U133A, gcrma* (http://biogps.org/dataset/1/)] [4], almost exclusively in astrocytes. GFAP is also present in the PNS along peripheral nerve fiber tracks, such as the sciatic nerve. In this case, GFAP is localized to non-myelinating Schwann cells that are believed to be functionally similar to astrocytes [5,6]. In addition, GFAP can be found in the glial cells of the ENS [2,3]. Such subepithelial glial cells have a trophic and supporting function of the intestinal epithelial cells and neurons. In addition, as GFAP-bearing glial cells are immediately associated with the enteric neurons and their nerve fibers in the submucosal and muscle layers of the gut, they are perfectly positioned to exert a neuromodulation function [for example, by active uptake of extracellular neurotransmitters by glial cell surface neurotransmitter (NT) transporters and subsequent degradation]. The enteric glial cell surface also has receptors for various NTs including purine P1 and P2 receptors that are responsive to neuron-released nucleotides and adenosine [7]. Since enteric neurons regulate gut motility and mucosal secretion, GFAP-bearing glial cells are thought to indirectly influence such processes as well. GFAP levels in enteric glial cells are also responsive to proinflammatory signals such as interleukin-6 (IL-6) or bacterial endotoxin LPS [8]. Similarly, GFAP and glial derived neurotrophic factor (GDNF) levels are highly upregulated in the mucosal plexus in the colon of patients with inflammatory bowel disease (IBD) [9]. In particular, GFAP might be an excellent index of the enteric gliosis response to the more severe forms of IBD (such as ulcerative and infectious colitis) over Crohn's disease. Intriguingly, mouse enteric glia can undergo neurogenesis in response to injury [3]. In addition, enteric GFAP levels and phosphorylation are also increased in Parkinson's disease (PD) patients [10]. Further investigation is needed to understand the possible implications of this finding in PD patients.

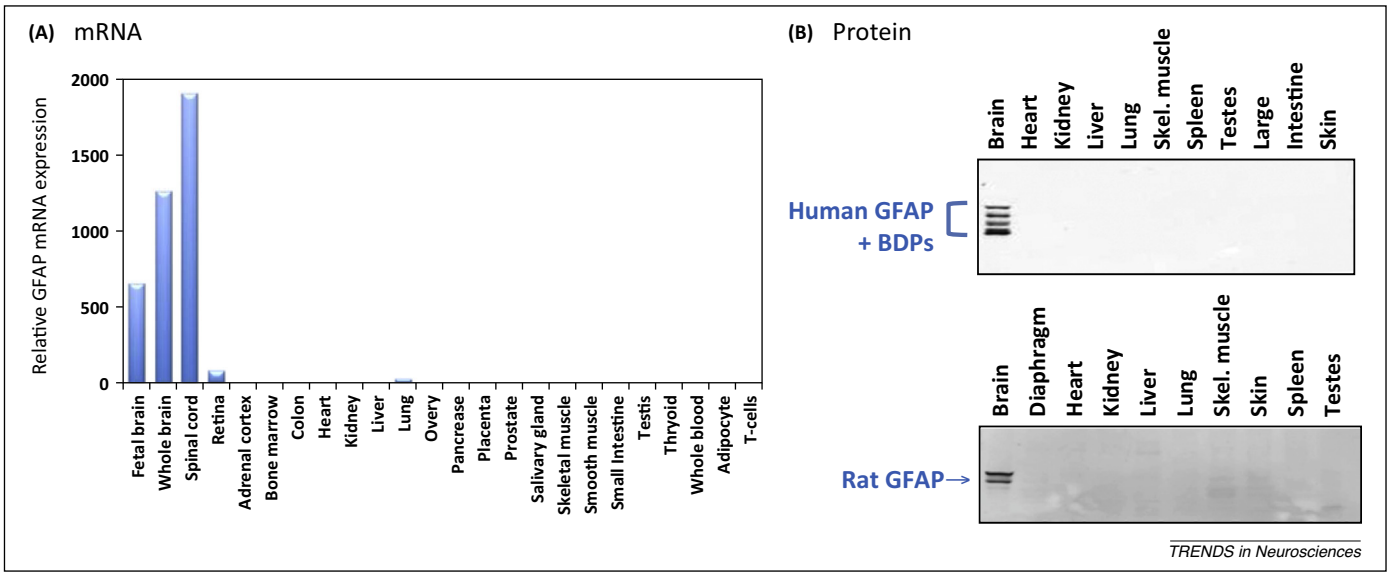

Figure 1. Glial fibrillary acidic protein (GFAP) tissue specificity. **(A)** GFAP mRNA expression in human tissue and cells based on the BioGPS database [Su, A., ed. (2012) *Dataset: GeneAtlas U133A, gcrma* (http://biogps.org/dataset/1/)]. **(B)** GFAP expression in various human and rat organs and tissues. Human GFAP was extensively truncated to 48–38 kDa bands, most likely due to postmortem proteolysis, while rat GFAP mainly exists in brain as 50–48 kDa form [4].

GFAP structure and function

GFAP is a key IF III protein responsible for the cytoskeletal structure of glial cells and for maintaining their mechanical strength, as well as supporting neighboring neurons and the blood–brain barrier (BBB) [1]. GFAP is structurally similar to other non-epithelial IF members (class III), including vimentin, desmin, and peripherin, and has head, rod, and tail domains. Activated astrocytes take on the morphology of thickened and elongated processes and GFAP – through its involvement in the IF network – is critical in the maintenance of such structure. In the most abundant isoform, GFAP-α, the head coil domain is followed by the rod domain comprising four coils (1A, 1B, 2A, 2B) flanked by three linker regions (1, 1,2, and 2, respectively) (Figure 2A). Such a structural organization is highly conserved among class III IF proteins [11]. Crystal 3D structure is currently not available, but through homology modeling using vimentin as a template a 3D structure of GFAP can be visualized (Figure 2B) [12]. Like other IF proteins, GFAP monomers transition to filamentous form by assembling first into dimers in parallel formation, followed by antiparallel assembly into tetramers, octamers, and so on (Figure 2C) [13]. The N-terminal head domain is critically important for filament elongation (including the M_1ERRRITSARRSY$_{14}$ motif), while the C-terminal tail domain is also important in facilitating oligomerization [14].

Isoforms/splice variants

GFAP is encoded by a single gene mapped to human chromosome 17q21. To date, there are ten isoforms/splice variants identified (Figure 3). GFAP-α (Isoform 1) is the predominant isoform in brain and spinal cord, but is also present in the PNS [15], and has the classic 432 residues (protein accession # NP_002046.1) with full usage of the nine exons within the GFAP gene [16]. GFAP-δ, also called GFAP-ε, (Isoform 2) (NP_001124491.1) is preferentially expressed by neurogenic astrocytes in the subventricular zone [17–21]. GFAP-δ includes the usage of an intron before exon 8 and has an alternative C-terminal and 431 residues. This unique C-terminal of GFAP-δ binds specifically to presenilin (PS) 1 and 2 proteins in yeast two-hybrid screening [19]. Presenilins are also involved in Notch signaling and may play a critical role in committing astrocytes into the GFAP$^+$ phenotype or neurogenic phenotype. It is tempting to suggest that presenilin–GFAP-δ interaction might be a regulatory mechanism of the Notch signaling pathway in astrocyte cell-fate determination [19]. Obviously further work is needed to clarify the physiologic and/or pathophysiologic significance of such an interaction. Enhanced GFAP-δ/ε expression in human astrocytic tumor has been reported [22]. GFAP-κ is the third isoform (Isoform 3) (NP_001229305.1); it includes an intron before exon 8 and an alternative shortened C-terminal and has 328 residues [23]. GFAP-α, GFAP-ε, and GFAP-κ are the three most well-characterized isoforms of GFAP. Of interest is that mRNA expression levels of all three isoforms gradually increase during development of the embryonic pig brain [23].

GFAP-β is highly expressed in non-myelinated Schwann cells in the PNS [24,25]. It includes the usage of a sequence before exon 1; the exact start site is currently unknown and has at least 432 residues. mRNA and protein levels of GFAP-β are apparently induced following neural injury [18]. GFAP-γ also includes the sequence before exon 1, but with exon 1 omitted, and includes an intron before exon 2. Again, its exact start site is currently unknown, but its length is predicted to be less than 432 residues. GFAP-γ mRNA is enriched in the corpus callosum and is also present in bone marrow and spleen [26]. GFAP-ζ is formed by the inclusion of the intron before exon 9 and has >438 residues [20] (Figure 3).

There are also four isoforms of GFAP, collectively called GFAP+1, reflecting its single-nucleotide frameshift-mediated variant formation. They are found in a subset of astrocytes throughout the brain. GFAP+1 includes:

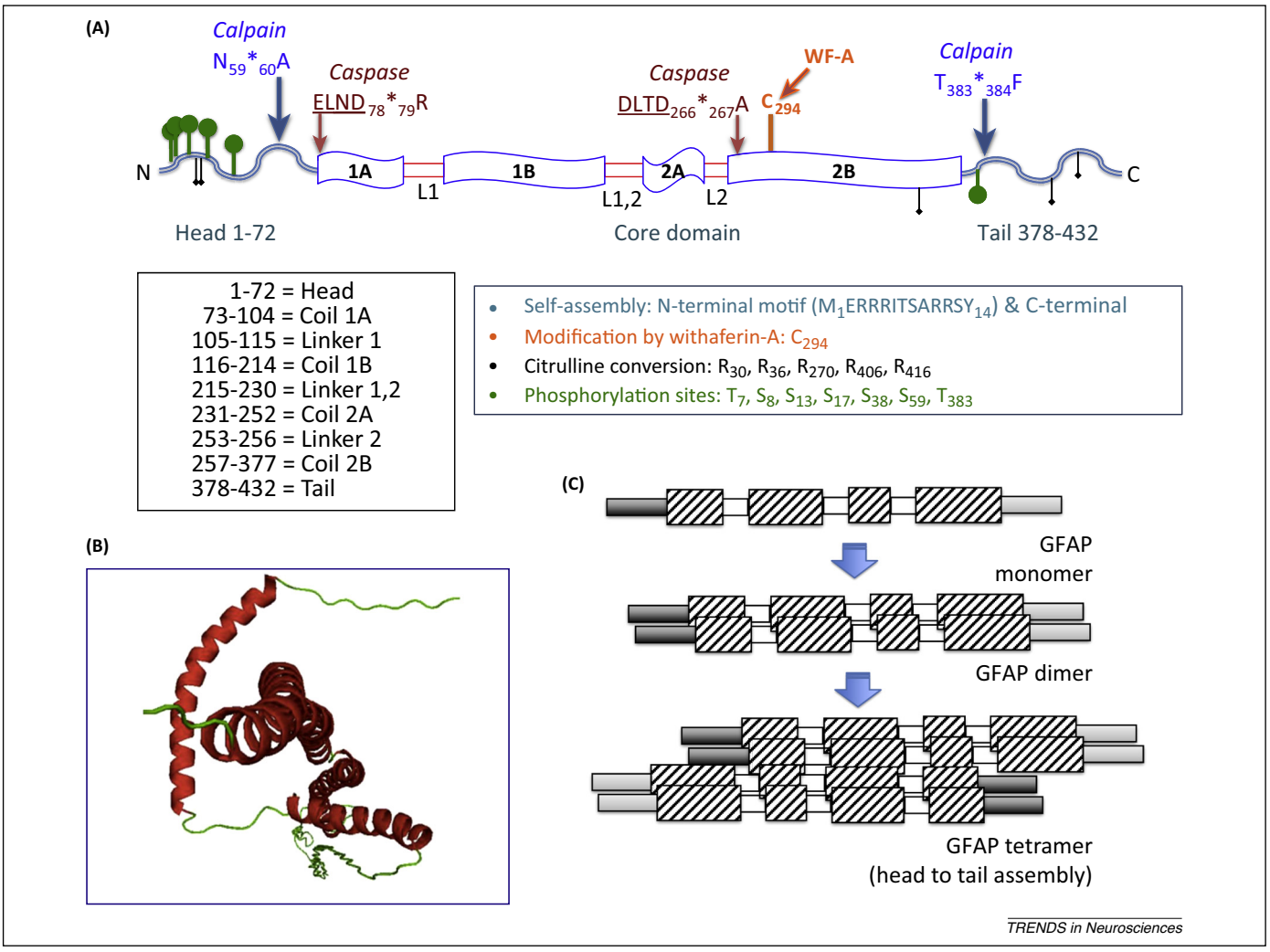

Figure 2. Glial fibrillary acidic protein (GFAP) structure and assembly. **(A)** Linear structure, functional domains, and key modifications. **(B)** 3D GFAP structure. Adapted from [12]. **(C)** Proposed GFAP dimer and tetramer assembly and oligomerization model. Adapted from [13].

GFAPΔEx6, with a skipped exon 6 and lacking Coil 2B within the core region (347 residues); GFAPΔ164, which has shortened exons 6 and 7 and lacks Coil 2B (366 residues); GFAPΔ135, which has shortened exon 6 and lacks Coil 2B (374 residues); and GFAPΔEx7, which has skipped exon 7 (418 residues) (Figure 3). The detailed structures of these isoforms are also described by Middeldorp and Hol [16]. Interestingly, in Alzheimer's disease (AD) brains, astrocytes near amyloid plaques have increased staining of both GFAP-α and GFAP-δ while GFAP+1 was found to be limited to a subset of astrocytes with long processes, with its numbers increased during the progression of AD [26]. Also, three GFAP+1 splice forms (GFAPΔ135, GFAPΔEx6, and GFAPΔ164) can be found in the pyramidal neurons of the hippocampus of AD and Down's syndrome patients [27]. The implication of such neuronal expression of GFAP remains unknown. Lastly, immunohistochemical evidence shows that GFAPΔEx6- and GFAPΔ164-positive astrocytes are found elevated and localized in focal lesions associated with chronic epilepsy; however, they appear far outnumbered by the dominant GFAP-α-positive astrocytes in the same lesioned regions [28]. Lastly, as many of these isoforms of GFAP have alternative N termini (β, γ) or alternative (δ/ε, κ, ζ) or shortened C termini (GFAP+1), it is tempting to suggest that they might have significant effects on GFAP filament assembly (Figure 3).

GFAP mutations/SNP and Alexander disease

GFAP is also a target for a SNP resulting in Alexander disease [29–32]. Several mutations are found mainly in the coding regions of the GFAP gene (L47; C79; H79; E223, H239; A244, R258, C289; D295 and R416), but a few mutations are found in the promoter regions (Table 1, top). These mutations were suggested to be 'gain-of-function' mutations [33], as GFAP knockout mice do not duplicate the Alexander disease phenotype [34,35]. The mutant GFAPs show a range of competency in IF assembly, from (i) fully able to assemble into full-length (10 nm) IFs, to (ii) capable of polymerization but not able to extend to full-length IFs, to (iii) not able to polymerize at all. In addition, mutated GFAPs are more prone to aggregate formation, producing astrocytic inclusions (called Rosenthal fibers) found in Alexander disease brains [30]. Messing *et al.* recently reviewed how various GFAP mutations are linked to the pathology of Alexander disease [33]. Some researchers believe that GFAP aggregates are toxic to astrocytes and thus might contribute to the astroglial degeneration and the subsequent white matter degeneration pathology

GFAP isoforms human accession no.		Localization	a.a.	Features
Gfap-α (Isoform 1) NP_002046.1		-Main isoform in astrocytes in CNS, -Also in schwann cells PNS	432	Predominant isoform in brain
Gfap-β		-High levels in PNS- schwann cells	>432	-Included sequence before exon-1; -Exact start site unknown
Gfap-γ		-Corpus callosum -mRNA found in brain, bone marrow, spleen	<432	-Included sequence before exon-1, omission of exon-1; included intron before exon-2 -Exact start site unknown
Gfap-δ /GFAP-ε (Isoform 2) NP_001124491.1		-Preferentially expressed by neurogenic astrocytes (SVZ) -Potential binding to presenilins	431	-Included intron before exon-8, alternate C-terminal
Gfap-κ (kappa) (Isoform 3) NP_001229305.1		-Transcripts can be found in mouse cortex, striatum and cerebellum	438	-Included intron before exon-8; alternate C-terminal
Gfap-ζ (zeta)		-Newly identified isoform	>432	Included intron before exon-9
GFAP +1	GFAPΔEx6	-Collectively called GFAP+1 reflecting its frameshift mediated variant formation -Subset of astrocytes throughout the brain; -- Implicated in AD, downs, epilepsy	347	Skipped exon-6; lacking coil 2B -Exact start site unknown
	GFAPΔ164		366	-Shortened exon-6, 7; lacking coil 2B -Exact start site unknown
	GFAPΔ135		374	-Shortened exon-6; lacking coil 2B
	GFAPΔEx7		418	-Skipped exon-7 -Exact start site unknown

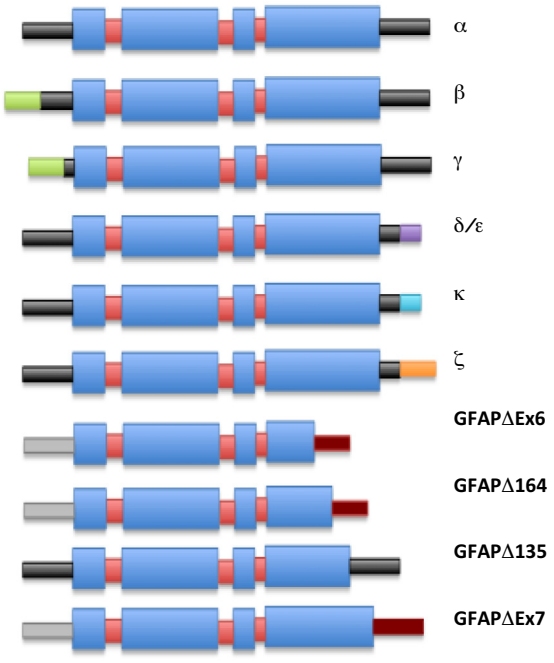

Figure 3. Known glial fibrillary acidic protein (GFAP) isoforms and features. Left: Description of key features of GFAP isoforms. Right: Schematic comparison of key GFAP isoforms in linear models.

observed in Alexander disease. Other researchers believe that Rosenthal fiber formation might simply be a reflection of excessive GFAP aggregate accumulation in dysfunctional astrocytes [33] (Box 1). Lastly, caspase-cleaved N-terminal GFAP fragments might contribute to GFAP aggregate formation in Alexander disease [36].

GFAP PTMs

GFAP is also subject to numerous PTMs (Table 1 and Figure 2). GFAP is highly regulated by protein kinases [such as protein kinase A (PKA), calmodulin-dependent protein kinase II (CAMPKII), and PKC], with many phosphorylation sites mapped to the N-terminal domain: T7 (PKA), S8 (PKA, PKC, cdk2), S13 (CAMPKII, PKA, PKC), S17 (CAMPKII), S38 (CAMPKII, PKA, PKC), and S289 (CAMPKII) (Figure 2 and Table 1) [13,37–40]. One of the key phosphorylation pathways of GFAP appears to involve the G protein-coupled mGluR receptor leading to calcium influx and CAMPKII activation [39,41]. As glutamate is a major excitatory neurotransmitter in the brain, such a mechanism might be involved in the crosstalk between neurons and glial cells [41]. In addition, since most of these phosphorylation sites reside at the N-terminal head domain, phosphorylation of GFAP has a negative effect on filament assembly. GFAP phosphorylation is elevated after hypoxia–ischemia in neonatal pig brain [37].

GFAP was recently identified as endogenously citrullinated; that is, several arginine residues undergo deamination to citrulline (R30, R36, R270, R406, and R416) [42]. The exact extent of citrullinated GFAP is not fully understood and how it alters GFAP function has not been elucidated. However, citrullinated protein epitopes are implicated in evoking autoimmune responses [43,44] which could be relevant as GFAP is a dominant autoantigen in certain neurological disorders (see below) [4]. Several lysine residues are also found to be subject to differential acetylation (K89, K153, K189, K218, K259, and K331) in the spinal cord of amyotrophic lateral sclerosis patients

Box 1. Outstanding questions

- Does GFAP citrullination and/or acetylation play a role in evoking autoimmune response to GFAP after brain injury or in other neurological disorders?
- What is the exact mechanistic relationship between aggregates of GFAP (Rosenthal fibers), astrocytic degeneration, and white matter degeneration in Alexander disease?
- How might the expression of different isoforms of GFAP produce different astrocyte responses to neuroinjury and/or other neuroperturbation?
- What are the differentiating utilities in monitoring intact GFAP versus GFAP-BDPs in biofluid following brain injury? For example, do the CSF or blood levels of GFAP-BDPs better reflect the extent of astrocyte injury post-TBI than the levels of intact GFAP?
- If specific GFAP-binding or GFAP-inhibiting pharmacological agents can be developed, will they be useful in treating neuroinjury conditions or other neurological disorders (e.g., Alexander disease)?

Table 1. GFAP modifications and modulators

Known modifications of GFAP			
Modification	*Site*	*Effects*	*Refs*
Phosphorylation	T7 (PKA) S8 (PKA, PKC, cdk2) S13 (CAMPKII, PKA, PKC) S17 (CAMPKII) S38 (CAMPKII, PKA, PKC) S289 (CAMPKII)	Inhibition of GFAP polymerization	[37–41]
Citrullination	Arginine deimination to citrulline at R30, R36, R270, R406, and R416	Altered GFAP conformation; increased autoimmune response	[42]
Acetylation	Acetylated lysine residues at K89, K153, K189, K218, K259, and K331	Found in ALS spinal cord (currently unknown effects of acetylation)	[45]
Proteolysis	Calpain sites (major): 69–70; 383–384 (BDP of 44—38 kDa) Caspase sites: 78–79, 266–267 (BDPs of 30 kDa, 20 kDa)	Disruption of IF elongation BDP appears gliatoxic	[46–53]
SNP	Promoter region; L47; C79; H79; E223, H239; A244, R258, C289; D295; R416	Formation of GFAP mutant aggregates (Rosenthal fibers) and causing Alexander disease	[31–33]
GFAP modulators			
Modulating molecule	*Chemical class*	*Mode of action and effects*	*Refs*
Inducers			
Thyroid hormone, glucocorticoids	Nuclear-receptor hormones	GFAP gene transcriptional activation	[61,62]
CNTF (FGF and TGFβ1)	Growth factors	GFAP gene transcriptional activation	[59,60]
LPS	Bacterial endotoxins	GFAP gene transcriptional activation	[64,68]
GDNF and neurturin	Glia-derived trophic factor	Promote GFAP gene transcriptional activation or stabilize GFAP levels	[73,74]
Suppressors/inhibitors			
S100b	Glial protein	Binds and inhibits GFAP phosphorylation, promotes disassembly of IF	[71,72]
WF-A	Steroidal lactone (from Winter cherry)	GFAP-covalent modification of Cys-294; inhibition of IF function	[78,79]
Prosaptide	N terminal of prosaposin	Downregulation of GFAP expression (precise mode of action unknown)	[75–77]
Ibudilast (AV411)	Pan-PDE inhibitor	Downregulation of GFAP expression (precise mode of action unknown)	[67,69,80]
Clomipramine (Clofranil)	Tricyclic antidepressant	Downregulation of GFAP expression (precise mode of action unknown)	[81]
Aspirin/acetylsalicylic acid	Cox-1 inhibitor	Downregulation of GFAP expression (5 mM *in vitro*) (precise mode of action unknown)	[82]
Curcumin	Curcuminoid (from turmeric)	Downregulation of GFAP expression (precise mode of action unknown)	[83]

(Figure 2 and Table 1) [45]. Such acetylation is spread across the whole GFAP, but its effects on GFAP structure and functions are currently unknown and warrant further investigation.

GFAP proteolysis/fragmentation in cultured astrocytes was noted, in some early reports, to be mediated by the calcium-activated protease calpain [46,47]. Increases in the fragmented form of GFAP have also been found in the spinal cord of patients with amyotrophic lateral sclerosis (ALS) [48]. We recently documented that GFAP is highly vulnerable to calpain-mediated truncation at both the C and N terminals. This results in a series of truncated GFAP breakdown products (BDPs) (38–44 kDa) compared with the 50-kDa intact protein during glial cell challenge *in vitro* [49,50] or in experimental brain injury in rodents as well as in cerebrospinal fluid (CSF) from TBI patients [4,49,50]. These fragments can also be observed in post-mortem human brain preparations (Figure 1). Similarly, we also found that GFAP-BDP can be observed in rodent and human CSF after spinal cord injury (SCI) [51,52]. The main calpain cleavage sites have been mapped to N59–A60 and T383–F384, although there are likely to be alternative cleavage sites near them. Regardless, the 'limit fragment' (~38 kDa) with both N- and C-terminal truncation leaves essentially the rod domain intact but the head and tail domains removed. It is thus predicted that such GFAP-BDPs would be unable to assemble into filaments. Caspase can also fragment GFAP in proapoptotic conditions, producing a shortened N-terminal fragment in AD brain (about 20 kDa) [53] at the $DLTD_{266}$–A_{267} site, which is located at the beginning of Coil 2B (Figure 2). Based on our own data, we also identified a second cleavage site at $\underline{ELND}_{78}{}^{*}{}_{79}R$ between the head and rod domains (Figure 2). Chen *et al.* recently demonstrated that GFAP might be cleaved by caspase-6 and that the C-terminal GFAP (C-GFAP) fragment (24 kDa) is unable to assemble

into filaments [36]. The 26-kDa N-terminal GFAP (N-GFAP) can form filamentous structures of variable length but is prone to forming filamentous aggregations [36]. However, in experimental TBI and in human TBI CSF, calpain-mediated GFAP fragments (44–38 kDa) appear to predominate over those produced by caspase [4,49].

The role of GFAP in astrocyte activation (astrogliosis) and GFAP inducers/activators

Astroglial cells respond to brain injury and other neuroperturbative conditions by undergoing 'reactive astrogliosis', a process whereby astroglial cells undergo cellular hypertrophy [increase of size and protein (GFAP) expression] and proliferation (increased number of glial cells) [1]. TBI itself, and its associated neuroinflammation, cause activation (and proliferation) of astroglial cells in damaged areas and a concomitant increase in GFAP levels [54]. Importantly, since GFAP, together with vimentin, is the key component responsible for the assembly and extension of the IF inside the astrocytic processes, it is believed that GFAP induction is critically important for the formation of the extended and thickened astrocytic processes observed in reactive gliosis. An increase in GFAP is a prominent feature of TBI and degenerative diseases such as PD and AD [4,55–58]. Different locations around the lesion site may exhibit different severities of gliosis; for example, a glial scar at the location of damaged tissue may be surrounded by areas with less severe astrocyte proliferation or hypertrophy. Diffuse traumatic injury can result in diffuse or more moderate gliosis without scar formation. Activated astrocytes (with highly expressed GFAP) are found to surround amyloid and neurite plaques in AD. Astrocytes in culture can also be used to study glial activation and GFAP induction. It is generally regarded that certain levels of post-injury gliosis might be beneficial to the recovery process following brain injury while excessive gliosis and its associated neuroinflammatory responses will have a negative impact on brain structural and functional recovery [57]. GFAP knockout mice were found to be essentially normal developmentally. In addition, post-SCI axonal sprouting and regeneration appear largely unaffected in GFAP$^{-/-}$ mice [34]. These results indicated a possible compensatory mechanism involving the related vimentin protein [34]. However, in a separate study with a peripheral nerve crush model, GFAP$^{-/-}$ mice showed defective Schwann cell differentiation and delayed nerve regeneration [35]. Again this is consistent with the concept that post-injury GFAP induction and associated reactive gliosis might promote neuroregeneration.

Numerous growth factors, such as ciliary neurotrophic factor (CNTF), fibroblast growth factor (FGF), and transforming growth factor beta (TGFβ), can induce GFAP gene transcription activation [59,60], leading to increased GFAP levels (Table 1 and Figure 4A). Nuclear-receptor hormones (thyroid hormone, glucocorticoids) can also activate GFAP transcription [61]. The effect of thyroid hormone might also be mediated via activation of the ROCK pathway [62]. These hormone- and growth factor-based GFAP gene regulators are through to be potentially important in the induction of mature astroglia formation. However, LPS, via the production of nitric oxide, is also a robust activator of cultured glial cells and induction of GFAP gene transcription [63]; it is likely that LPS activates Toll-like receptor 4 (TLR-4) and possibly CD14 in astrocytes [64]. Systemic administration or intracerebral injection of LPS also leads to gliosis and upregulation of GFAP

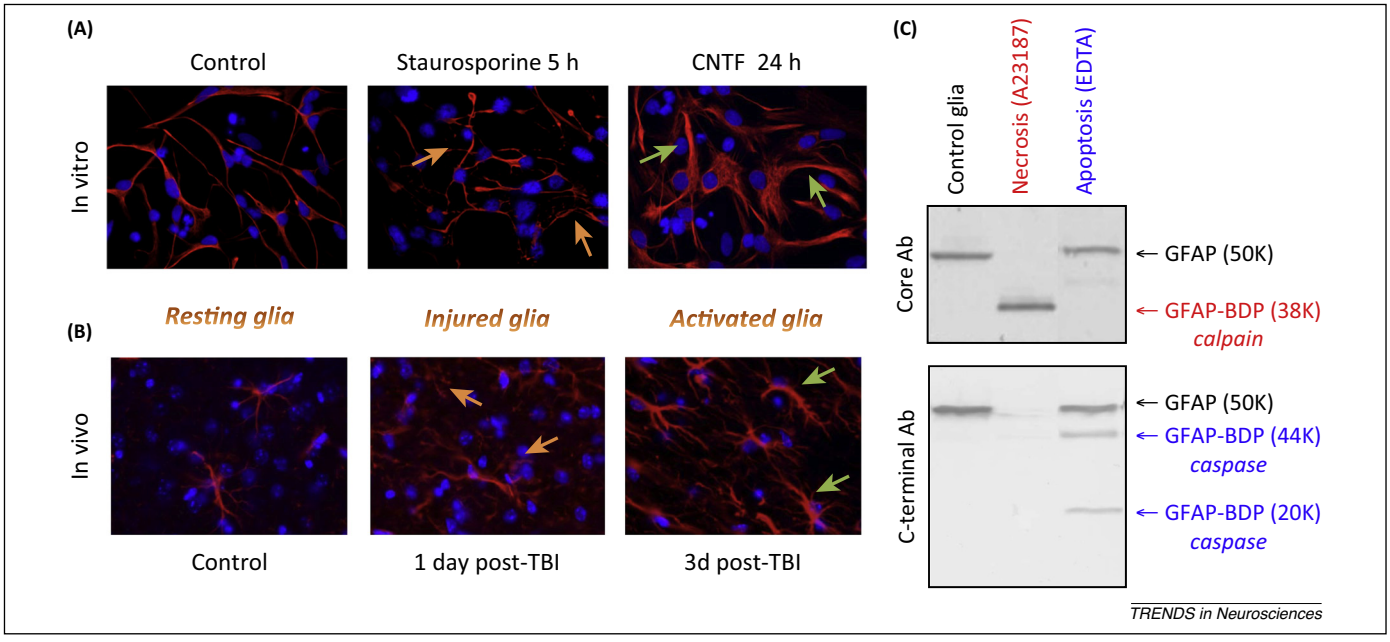

Figure 4. Glial fibrillary acidic protein (GFAP) patterns in injured and activated glial cells *in vitro* and *in vivo*. **(A)** Rat GFAP staining of resting primary glial cells, staurosporine (0.5 μM, 5 h)-injured glial cells, and ciliary neurotrophic factor (CNTF) (200 nM for 24 h)-activated glial cells. **(B)** Naïve (control) rat cortex and 24 h after experimental traumatic brain injury (TBI) showing injured glial cells or 3 days after TBI showing activated glial cells. GFAP was stained red while the counterstain was DAPI for nuclear DNA (blue). **(C)** Anti-GFAP immunoblotting of control glial cell lysate shows intact GFAP of 50 kDa with two anti-GFAP antibodies. On induction of necrosis (by 10 μM A23187) or caspase-dominant apoptosis (by 5 mM EDTA) [50,112], core-directed antibody shows a calpain-mediated 38-kDa limit GFAP breakdown product (BDP) after A23187 treatment while C-terminal antibody detects two district caspase-mediated GFAP-BDPs of 44 kDa and 20 kDa after EDTA treatment. {Results shown in both (A) and (B) are from a study published by us [4], but were not shown as representative examples in a primary publication.}

and neuroinflammation in live animals [65–70]. Thus, LPS-based GFAP upregulation is more in line with astrogliosis formation.

GFAP is also modulated endogenously by calcium-dependent binding to EF-hand S100β. On binding, S100β promotes disassembly of IFs [71]. S100β binding leads to inhibition of GFAP phosphorylation, which might be the mode of action that leads to the disassembly of IFs [72]. GDNF and neurturin (another glia-derived trophic factor) have also been shown to be glia protective [73,74]. Thus they might serve as autoregulating factors that either promote GFAP gene transcriptional activation or stabilize GFAP levels (Table 1).

GFAP suppressors and glia-targeting therapeutic agents

Several therapeutic agents can inhibit glial cell function or suppress GFAP expression (Table 1). An interesting compound is prosaptide, which is a 14-mer (Thr-*D*-Ala-Leu-Ile-Asp-Asn-Asn-Ala-Thr-Glu-Glu-Ile-Leu-Tyr) derived from the neurotrophic and glia-tropic N terminal of the human glycoprotein prosaposin [75–77]. Prosaptide is also known to cross the BBB to exert its GFAP-suppression effects. Second, several drug-like agents have reported effects in suppressing either GFAP expression and/or gliosis induction. These include Withaferin-A (WF-A), ibudilast, clomipramine, aspirin, and curcumin. WF-A is a steroidal lactone that binds to and thus inhibits GFAP and the related IF protein vimentin [78,79]. It was first isolated from the Ayurvedic medicine Winter cherry and is a cancer drug due to its antiangiogenic effects. It was found that WF-A covalently modifies the single Cys-294 of GFAP (and a homologous cysteine residue in vimentin protein) leading to inhibition of IF function of GFAP and filament disassembly [79] (Figure 2 and Table 1). WF-A can attenuate GFAP levels and glial cell activation and appears to be BBB permeable as it exerts its central effects in a model of retinal gliosis [79]. Ibudilast is an anti-inflammatory drug currently used mainly in Japan as an asthma treatment. Ibudilast is a broad-spectrum phosphodiesterase inhibitor (PDE) that also inhibits methamphetamine-induced GFAP upregulation and gliosis and attenuates methamphetamine self-administration and relapses [67,69]. Ibudilast also appears to attenuate mechanical allodynia in rat models of neuropathic pain [80]. The tricyclic antidepressant clomipramine is also found to robustly suppress GFAP levels *in vivo* [81]. Aspirin/acetylsalicylic acid (5 mM *in vitro*), although having various anti-inflammatory actions including inhibition of cyclooxygenase 2 (COX-2), was unexpectedly found to also inhibit GFAP upregulation and glial cell activation via a nuclear factor kappa B (NF-κB) pathway *in vitro* [82]. Curcumin might also have beneficial effects by downregulation of GFAP expression in an *in vitro* model of Alexander disease [83]. Although the exact modes of action (direct binding or regulation of protein levels) for GFAP attenuation by prosaptide, ibudilast, clomipramine, aspirin, and curcumin are presently unknown, they might still be useful in studying the role of GFAP in neurodisease models. Screening efforts are under way to find additional druggable molecules that can either specifically suppress or reverse GFAP aggregate formation (for Alexander disease) or suppress GFAP expression (for gliosis), including several novel chemical candidates [75,81]. Molecular modeling and compound docking *in silico* is also being applied for such drug discovery efforts [12]. However, at present it is unclear whether the binding of small molecules to GFAP monomers is sufficient to: (i) reduce steady-state levels of GFAP; (ii) inhibit GFAP polymerization and thus astrogliosis; or (iii) destabilize intracellular GFAP aggregates (Box 1).

GBPs as markers for glial cell injury

Neurotrauma conditions are often associated with neuronal injury or death. However, since astrocytes are a major cell type in the brain we proposed that they are also subject to mechanical or chemical injury shortly after neurotrauma and in neurodegenerative disorders. Our recent work shows that 'glial injury' is a key pathologic event during the acute/subacute phase of neurotrauma (in animal models of TBI and SCI, as well as in human TBI/SCI CSF samples), as aided by our newly identified glial injury signature; that is, the proteolytic conversion of the intact GFAP (50 kDa) into GFAP-BDPs of 44–38 kDa by calpain [4,49,51,52,84,85] (Figure 4B). Similarly, GFAP is degraded into GFAP-BDPs by calpain proteases in cultured glial cells challenged with cytotoxins (staurosporine or calcium ionophore) [50] (Figure 4A). Caspase protease can also contribute to GFAP proteolysis, to a lesser extent (see above) [4].

GFAP as a biomarker protein for acute CNS injury and other neurological conditions

Increasing evidence also suggests that GFAP and GFAP-BDPs might be useful tools as biofluid-based markers for numerous neurological conditions. The overall concept is that brain injury causes the release of GFAP-BDPs and, to a lesser extent, full-length GFAP from injured astrocytes into the interstitial fluid (ISF)/extracellular fluid, where these proteins equilibrate into the subarachnoid CSF compartment and are then released to the circulating blood by direct venous drainage (glymphatic pathway) [86] or continue to follow the CSF flow and eventually enter the circulation by diffusing past the (possibly compromised) BBB (Figure 5). An advantage of GFAP as a brain biomarker is that it shows strong brain specificity and high expression levels in the brain (Figure 1). There are over 1.7 million cases of TBI in the USA each year [87]. TBI is categorized by the Glasgow coma scale (GCS) or CT abnormality as severe (GCS 3–8, cranial CT abnormal; ~10%), moderate (GCS 9–12, CT abnormal; ~5–10%), or mild (GCS 13–15, CT normal). Additionally, impact or blast overpressure wave-induced TBI is a signature injury of recent warfare among USA military personnel [88,89]. In penetrating TBI as well as overpressure blast wave-induced brain injury in rats, GFAP levels are elevated in CSF and/or serum at 4–24 h after injury [50,90]. Emerging evidence from others and us also shows robust release of GFAP and its BDPs into CSF and blood compartments hours and even days after the initial severe TBI event [91,92]. GFAP levels are linked to CT pathology and outcome in these subjects. GFAP blood levels can also help predict secondary insults after severe TBI [93]. Since moderate TBI is often difficult to distinguish from mild TBI in

Neuro-disease/ Disorder	Species, biofluid type	Temporal profile	Key refs.
Hemorrhagic stroke; ICH	Human serum	< 4 h	[96-100]
Cerebral ischemia	Human ischemic stroke Rat MCAO	24-72 h	[97-98]
Severe TBI	Human CSF serum Rat CSF, serum	6 h – 24 h	[90-93]
Mild TBI	human serum, plasma	< 24 h	[85-86]
Spinal cord injury	Human, CSF, serum rat CSF, serum	< 24 h	[52-53]
Glioma / Glioblastoma	Human biofluid	-	[105-106]

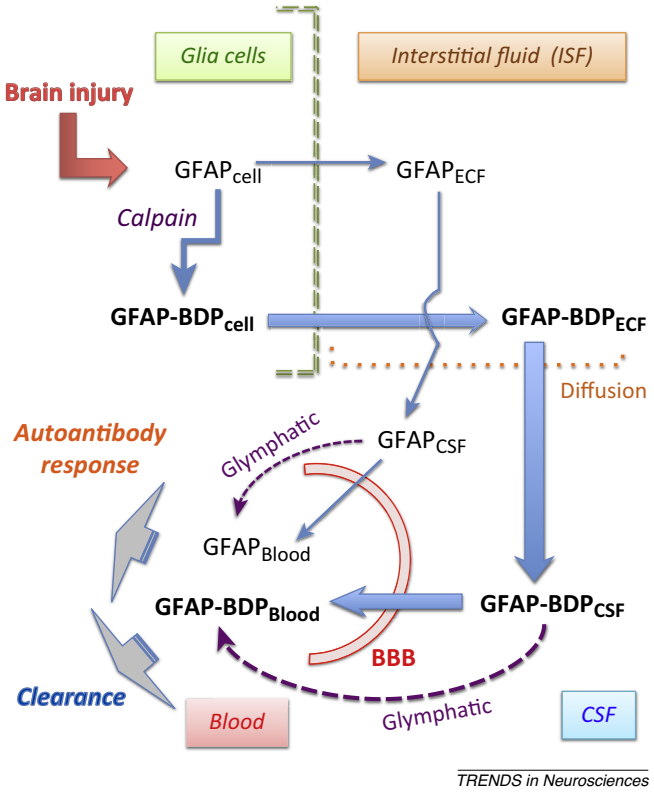

Figure 5. Release of glial fibrillary acidic protein (GFAP) and GFAP breakdown products (BDPs) into biofluid as acute CNS injury biomarkers. Left: List of neurodiseases and disorders in which GFAP and/or GFAP-BDP is released into biofluid. Right: Schematic showing how GFAP-BDPs are generated after traumatic brain injury (TBI) and how both GFAP-BDPs and, to a lesser extent, intact GFAP are released into interstitial fluid (ISF)/extracellular fluid. From there, these proteins diffuse into the subarachnoid cerebrospinal fluid (CSF) [86]. GFAP-BDPs/GFAP then either drain directly into the veins (glymphatic pathway, broken arrows) or continue to follow the CSF flow through the ventricles and then enter the circulation by diffusing through the blood–brain barrier (BBB). Blood-based GFAP and GFAP-BDPs can be detected as biomarkers but they also can serve as autoantigens, triggering an autoantibody response in a subset of patients.

the emergency room, there are two important studies that show that the blood levels of GFAP and/or its BDPs can be used to predict CT abnormality and thus can potentially differentiate mild versus moderate TBI [85,86]. Consistent with these reports, blood GFAP loads were also elevated in individuals undergoing breacher training and exposure to blast overpressure waves [94]. GFAP levels in both brain tissue and serum are also elevated in an animal model of blast overpressure wave-induced TBI combined with psychological stress [95].

There are two type of stroke, hemorrhagic and ischemic. The former is a result of intracerebral hemorrhage while the latter is due to blockage of a major blood vessel resulting in brain ischemia. It is important to differentiate the two, as tissue plasminogen activator (TPA) is currently the only FDA-approved treatment for ischemic stroke. TPA (usually given within 3–4 h post-injury) works by dissolving the existing blood clots in the brain and reducing further blood clot formation. However, TPA is contraindicated under cerebral hemorrhage conditions. Increasing evidence shows that GFAP might be a key marker that can distinguish the two. Several studies have shown that GFAP is released within 3–4 h following hemorrhagic stroke, while its release is delayed to 24–48 h post-injury in ischemic stroke [96–100]. Thus, early release of GFAP into blood is a clinically important differentiating indicator of hemorrhagic stroke over ischemic stroke. In addition, GFAP appears to be useful in tracking the progression or outcome of ischemic stroke [97,98] (Figure 5).

Taken together, these data strongly suggest that once a robust GFAP sandwich ELISA test becomes universally available, it will help realize the potential of GFAP as a biomarker for various forms of CNS injury. This is considered an important discovery as to date there are no FDA-approved *in vitro* diagnostic biofluid tests to monitor brain injury. Thus, it can be envisioned that a GFAP biofluid test used routinely in brain injury patient management will change medical practice. In addition, based on others' and our recent observations, GFAP-BDP formation and subsequent release (rather than intact GFAP) into biofluids appears intimately tied to astrocyte damage or cell death after brain injury (Figure 5). Thus we reason that the elevated levels of GFAP-BDPs in accessible biofluids (CSF, blood) could then be used to track the levels and kinetics of astrocyte cell damage following brain trauma. This is an under-investigated area that could be exploited for novel therapy development. In addition, from a clinical standpoint, it will be important to define whether there are any differentiating utilities in monitoring intact GFAP versus GFAP-BDPs in biofluid following brain injury (Box 1).

GFAP biomarker tests can also be further developed into 'theranostic' guides to help much-needed drug development for CNS injury, as FDA-approved therapies in this

area are almost nonexistent. For instance, drug treatment given after brain injury that is beneficial in reducing brain injury or promoting brain recovery should also reduce the biomarker load [101–103]. GFAP assay might also be useful as a neurotoxicity biomarker tool during drug development [104]. Additionally, since most gliomas (including glioblastoma) also express GFAP, monitoring of biofluid levels of GFAP might reflect such tumor formation or expansion [105,106].

GFAP or a PTM form of the protein might become an autoantigen in neurodegenerative conditions, autism, workers exposed to lead, or chronic cerebrovascular disorders [107–110]. We recently reported that about 40% of severe TBI patients unexpectedly show an immunodominant autoantibody response to GFAP and its BDPs 5–6 days post-injury [5]. Our hypothesis is as follows. TBI causes (i) protease-mediated GFAP-BDP formation in injured glial cells and (ii) subsequent release of such GFAP-BDPs in substantial quantities through the compromised BBB into the circulation. (iii) The combined effect is that GFAP-BDPs become accessible to and recognized by the immune system as non-self protein, triggering an autoantibody response in these vulnerable individuals. (iv) Autoantibody specifically targeting a major brain protein such as GFAP might trigger a persistent autoimmune attack on the CNS and negatively affect TBI patients' long-term outcome. Our findings of GFAP being an post-neurotrauma autoantigen have been extended to SCI [52]. It is also possible that citrullinated GFAP is preferentially recognized by the immune system as an autoantigen, as citrullinated proteins are identified as robust autoantigens in autoimmune diseases such as rheumatoid arthritis [44,45] (Box 1). It is important to further examine whether such an autoantibody response is long lasting and whether it leads to autoimmune attack on the nervous system, similar to multiple sclerosis. Interestingly, serum autoantibody to GFAP also might have utility in tracking glioma progression [111].

Concluding remarks and future directions

This review indicates the fascinating roles that GFAP plays in our nervous system. They include maintaining the structure and function of GFAP-bearing cells in the CNS, PNS, and ENS while also mediating astroglial cell activation in the event of nervous system injury. It also appears that the number of GFAP splice variants in humans is likely to exceed the ten isoforms identified to date, while the number of GFAP mutations that lead to Alexander disease is still growing. GFAP is also highly regulated both pre- and post-translationally and is a target of numerous pharmacological agents. Mounting clinical evidence also suggests that GFAP is arguably one of the most promising biofluid-based biomarkers, with diagnostic, prognostic, and even theranostic utilities in managing neuroinjuries and possibly other neurodiseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tins.2015.04.003.

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