Alexander disease GFAP R239C mutant shows increased susceptibility to lipoxidation and elicits mitochondrial dysfunction and oxidative stress

Álvaro Viedma-Poyatos, Patricia González-Jiménez, María A. Pajares, Dolores Pérez-Sala*

Department of Structural and Chemical Biology, Centro de Investigaciones Biológicas Margarita Salas, C.S.I.C., 28040, Madrid, Spain

ARTICLE INFO

Keywords:
- Neurodegeneration
- Mitochondrial dysfunction
- GFAP aggregation
- Protein lipoxidation
- Oxidative damage
- AxD cellular model

ABSTRACT

Alexander disease is a fatal neurological disorder caused by mutations in the intermediate filament protein Glial Fibrillary Acidic Protein (GFAP), which is key for astrocyte homeostasis. These mutations cause GFAP aggregation, astrocyte dysfunction and neurodegeneration. Remarkably, most of the known GFAP mutations imply a change by more nucleophilic amino acids, mainly cysteine or histidine, which are more susceptible to oxidation and lipoxidation. Therefore, we hypothesized that a higher susceptibility of Alexander disease GFAP mutants to oxidative or electrophilic damage, which frequently occurs during neurodegeneration, could contribute to disease pathogenesis. To address this point, we have expressed GFP-GFAP wild type or the harmful Alexander disease GFAP-GFAP R239C mutant in astrocytic cells. Interestingly, GFAP R239C appears more oxidized than the wild type under control conditions, as indicated both by its lower cysteine residue accessibility and increased presence of disulfide-bonded oligomers. Moreover, GFP-GFAP R239C undergoes lipoxidation to a higher extent than GFAP wild type upon treatment with the electrophilic mediator 15-deoxy- Δ12,14-prostaglandin J2 (15d-PGJ2). Importantly, GFAP R239C filament organization is altered in untreated cells and is earlier and more severely disrupted than GFAP wild type upon exposure to oxidants (diamide, H2O2) or electrophiles (4-hydroxynonenal, 15d-PGJ2), which exacerbate GFAP R239C aggregation. Furthermore, H2O2 causes reversible alterations in GFAP wild type, but irreversible damage in GFAP R239C expressing cells. Finally, we show that GFAP R239C expression induces a more oxidized cellular status, with decreased free thiol content and increased mitochondrial superoxide generation. In addition, mitochondria show decreased mass, increased colocalization with GFAP and altered morphology. Notably, a GFP-GFAP R239H mutant recapitulates R239C-elicited alterations whereas an R239G mutant induces a milder phenotype. Together, our results outline a deleterious cycle involving altered GFAP R239C organization, mitochondrial dysfunction, oxidative stress, and further GFAP R239C protein damage and network disruption, which could contribute to astrocyte derangement in Alexander disease.

1. Introduction

Alexander disease (AxD) is a devastating neurological disorder caused by \textit{de novo} mutations in the type III intermediate filament protein glial fibrillary acidic protein (GFAP), which is mainly expressed in astrocytes [1–3]. AxD GFAP mutations cause leukodystrophy, with progressive destruction of the white matter, and loss of neurons. Clinically, AxD symptoms may include seizures, megalencephaly, spasticity and cognitive impairment, and result in a fatal outcome [3,4].

GFAP forms the cytoplasmic intermediate filament network of mature astrocytes together with vimentin [5,6]. The GFAP monomer structure is integrated by a mainly \(\alpha\)-helical central domain (rod), flanked by intrinsically disordered head and tail domains (Fig. 1A). More than ten isoforms of GFAP have been identified that arise from alternative splicing and differ mainly in the C-terminal domain [2,7]. The most abundant and thoroughly studied isoform is GFAP\(\alpha\), which will be the subject of the present study. Like other type III intermediate filament proteins, assembly of GFAP into filaments is considered to proceed through the association of monomers into parallel dimers, the antiparallel association of dimers into tetramers, and the gathering of...
several tetramers into unit length filaments that elongate by head to tail engagement [8,9]. GFAP is known to heteropolymerize with vimentin in mature astrocytes, although the precise structure of the mixed filaments is not well understood [5]. The GFAP network in astrocytes is constituted by dynamic filaments that expand from the periphery of the nucleus towards the cell membrane, establishing interactions with multiple cellular structures. GFAP expression increases in brain disease and trauma [2], for which has been traditionally considered a marker of disease [10]. Indeed, GFAP plays an important role in the response to brain damage, and in reactive astroglia [11], a response of astrocytes to perturbations of the central nervous system implying cell activation, hypertrophy and proliferation [12]. Nevertheless, the role of GFAP in these processes may be complex. A protective role for the increased expression of GFAP after brain injury has been put forward through the limitation of the damaged area, although at later stages, this increase could have a negative counterpart by delaying regeneration [11]. Nevertheless, accumulating evidence from knockout animal models demonstrates that GFAP plays key roles in basal astrocyte functions and, therefore, in neuronal health and brain homeostasis [2,7]. In fact, GFAP is involved in astrocyte migration and proliferation and organelle positioning. Moreover, GFAP is required for maintenance of the blood brain barrier, influences glutamatergic neurotransmission and adult neurogenesis [2,7].

As for other intermediate filaments, GFAP assembly and regulation is finely tuned by posttranslational modifications. GFAP can be phosphorylated at multiple sites along its sequence, particularly at the head domain, which is associated with filament disassembly [13,14]. Glycosylation, acetylation and citrullination of GFAP have also been reported, in some cases in pathological contexts, such as amyotrophic lateral sclerosis and Alzheimer’s disease, suggesting that certain modifications could contribute to or be a consequence of the disease. GFAP is also the target for numerous oxidative and lipoxidative modifications, recently reviewed in Ref. [15]. These modifications are especially relevant since neurodegenerative diseases are often accompanied by neuroinflammation and oxidative stress [16], during which, increased oxidative and electrophilic species are generated [17,18]. Moreover, protein oxidation and lipoxidation can favor aggregation, which is a hallmark of many neurodegenerative diseases [17]. Importantly, GFAP is a major target of lipoxidation by 4-hydroxynonenal (HNE), and higher levels of HNE-modified GFAP have been evidenced in the frontal cortex of Down’s syndrome patients [19], who develop neuropathological features similar to Alzheimer’s disease, as well as in the cortex of patients of frontotemporal lobar degeneration [20] or Pick’s disease [21]. GFAP has also been reported to be particularly susceptible to lipoxidation by neurokets in normal brain cortex [22], and to undergo increased protein carbonyl formation in a variety of diseases (reviewed in Ref. [15]). Notably, an eight-fold increase in malondialdehyde-modified GFAP has been found in the brain cortex of Alzheimer’s disease patients, whereas vimentin was not modified [23]. Nevertheless, the sites and precise functional consequences of these modifications are not fully understood.

Type III intermediate filaments possess a conserved cysteine residue, which is the only cysteine residue of the protein in GFAP, vimentin and desmin. We have previously shown that the single cysteine residue of GFAP, C294, is the target for lipoxidation by the reactive lipid mediators known as cyclopentenone prostanoids (cyPG) in several cellular models, and this modification causes a disruption of the GFAP network with formation of protein aggregates and perturbation of the cytoskeleton [24]. Moreover, C294 is involved in crosslinking with both other GFAP monomers and vimentin in cells [24], as previously observed in vitro [25]. Notably, this cysteine residue is important for GFAP self-assembly as well as for heterooligomerization with vimentin, since the formation of an extended GFAP network is markedly delayed in primary astrocytes expressing a C294S GFAP mutant [24]. These observations illustrate that either single point mutations and/or modifications of key GFAP residues can have important functional consequences.

GFAP mutations causing AxD are most often point mutations which can occur at any segment of the protein, although certain sites, including R239, R79 and R416, appear more frequently mutated (Fig. 1A). The more than a hundred mutations of GFAP known to date are collected in a dedicated database at https://alexander-disease.waisman.wisc.edu. AxD GFAP mutations elicit a dominant gain of function in astrocytes [26], accompanied by an increase in GFAP expression, interference with normal polymerization, and aberrant accumulation in cytoplasmic aggregates known as Rosenthal fibers, containing GFAP and other proteins, which, interestingly, display lipoxidative damage [27]. The expression of mutant GFAP is associated with severe astrocyte dysfunction. Evidence obtained from various cellular and animal models has revealed the induction of a stress response, alterations in glutamate transport, proteasome activity and autophagy, multiple alterations in gene expression with increase in proinflammatory genes, organelle dysfunction, neuronal damage and death [28-31].

Being mainly point mutations, it is not yet clear how mutant GFAP causes the disruption of the network leading to such an extensive damage. There are indirect pieces of evidence from AxD models suggesting the occurrence of oxidative stress. An upregulation of Nrf2 and several of its targets, including enzymes involved in glutathione metabolism, has been observed in mouse models of the disease [32,33]. In these models, both overexpression and knockout of Nrf2 decreases the levels of GFAP [34,35], whereas overexpression of antioxidant enzymes modulates mutant GFAP toxicity in a fly model [36]. Remarkably, most of the AxD mutations described, including the frequent and severe mutation R239C, imply a change by a more nucleophilic amino acid, most often cysteine or histidine, which are more susceptible to oxidation and lipoxidation. Given the potential occurrence of oxidative stress in AxD and the antecedents on GFAP lipoxidation, we formulated the hypothesis that an increased susceptibility of GFAP AxD mutants to oxidative and/or lipoxidative modifications could contribute to AxD pathogenesis. To test this hypothesis, here we have employed a GFAP-deficient astrocytoma cell line in order to express GFAP wild type (wt) or R239C and explore the impact of the mutant protein on the assembly of the intermediate filament network, as well as its susceptibility to oxidative and electrophilic stress. Our results indicate that the GFAP R239C mutant is indeed more susceptible to oxidation and lipoxidation, and, importantly, that its expression in cells is sufficient to induce oxidative stress and mitochondrial alterations. Moreover, oxidants and electrophiles selectively increase aggregate formation and induce cytoxicity in cells expressing GFP-GFAP R239C. These results may contribute to understand the deleterious effect of GFAP R239C on the organization of the intermediate filament network in AxD through the instauration of a vicious cycle potentially involving oxidative stress and protein oxidation and unfolding.

2. Materials and methods

Materials - Cell culture media and supplements were from Gibco (Life Technologies). Astrocyte basal medium was from Cyagen. Fetal bovine serum (FBS) was from Sigma or Biowest. 15-deoxy-A12,14-PGJ2 (15d-PGJ2) and its biotinylated analog (15d-PGJ2-B) and 4-hydroxynonenal (HNE) were from Cayman Chemical. Iodoacetamide, biotinylated iodoacetamide (iac-B), diamide, bovine serum albumin (BSA), detergents and buffers were from Sigma or Merck. Primary antibodies used were anti-GFAP (Z 0334, Dako), anti-vimentin (sc-6260) and anti-TOM20 (sc-17764) from Santa Cruz Biotechnology, anti-actin (A2066, Sigma), and anti-GFP (#11814460001, Roche). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Dako. The fluorescent probes used for flow cytometry, namely, Mitofus Red, CellRho Deep Red, ThioTracker Violet, Tetramethylrhodamine Methyl Ester (TMRM) and MitoTracker Deep Red, were from ThermoFisher Scientific.

Cell culture and treatments - The U-251 MG and U-87 MG astrocytoma cell lines were obtained from the American Type Culture Collection (ATCC). Cells were authenticated by microsatellite
amplification (short tandem repeat (STR)-PCR profiling) at Secugen, S.L. (Madrid, Spain), and routinely checked for mycoplasma contamination at the Cell Culture Facility (CIB Margarita Salas). Sprague-Dawley rat primary astrocytes were from Cyagen. Cells were grown at 37 °C (Madrid, Spain), and routinely checked for mycoplasma contamination.

Concentration and purity was assessed by UV spectra using Nanodrop. DNA were purified with the EndoFree Plasmid Maxi Kit (Biotools). DNA was transformed into E. coli strain XL-10 Gold (Stratagene) in the presence of the corresponding selection antibiotic and isolation of the plasmids with the High-Pure Plasmid Isolation kit (Roche). Plasmids for transfection were mutated using the QuikChange XL Site-directed Mutagenesis kit (Agilent) using the sense and the corresponding antisense synthesized at the Protein Chemistry Facility (CIB Margarita Salas). Introduction of the desired mutation was confirmed by DNA sequencing (Secugen SL) after amplification using the E. coli strain XL-10 Gold (Stratagene) in the presence of the corresponding selection antibiotic and isolation of the plasmids with the High-Pure Plasmid Isolation kit (Roche). Plasmids for transfection were purified with the EndoFree Plasmid Maxi Kit (Biotools). DNA concentration and purity was assessed by UV spectra using Nanodrop.

Plasmids coding for GFP-GFAP R239H and R239G were obtained from Abvance Biotech. Transfections were carried out at 70% cell confluence in 35 or 60 mm dishes using 1 or 1.8 μg of DNA, respectively, and Lipofectamine 2000 (Invitrogen) for 5 h in antibiotic-free medium. After transfection, cells were kept in antibiotic-free medium for 48 h for recovery, at which time they were used for experiments as transiently transfected cells. To obtain stable transfectants, cells were cultured in the presence of selection antibiotic (500 μg/ml geneticin, G418, Gibco), for at least five passages after transfection. Stable expression of GFP-GFAP was confirmed by fluorescence microscopy. Transient transfections yielded higher levels of GFP-GFAP and were generally employed to assess incorporation of biotinylated electrophiles. Stable transfections yielded more moderate and homogeneous GFP-GFAP levels and were mostly employed for functional studies.

**Immunofluorescence, confocal microscopy and image analysis** - Cells grown on glass-bottom dishes (MatTek Europe) or on glass coverslips were fixed with 4% (v/v) paraformaldehyde (PFA) in PBS for 25 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min and incubated for 1 h with a blocking solution containing 1% (w/v) BSA in PBS. Subsequently, incubations with primary antibodies were carried out for 1 h, followed by washes in PBS and 1 h incubation with Alexa-conjugated secondary antibodies. Antibodies were diluted in blocking solution, at 1:1000 (v/v) for anti-GFP and at 1:200 (v/v) for all other antibodies. Nuclei were counterstained with 3 μg/ml DAPI in PBS for 15 min. For staining of f-actin, fixed and permeabilized cells were incubated with 4 μl Alexa-568 Phalloidin (Molecular Probes) in blocking solution. All incubations were performed at room temperature (r.t.). Cells were visualized in whole cells, cells expressing extended GFP-GFAP wt networks were used as standard to define acquisition parameters. When specified, images were acquired with the Lightning module of the Leica SP8 microscope.

Analysis and quantification of confocal images was carried out with ImageJ. The extension of the GFAP network was established as the ratio between the area occupied by detectable fluorescence from filamentos GFAP and the total cell area indicated by either the cortical f-actin signal or the cell contour. Regions of interest (ROI) were selected manually in total projections. Where indicated, GFAP perinuclear condensation was determined as the ratio of GFAP fluorescence intensities measured in the perinuclear (comprising a distance of 6 μm from the nuclear contour) and peripheral (within a distance of 6 μm from the plasma membrane) regions. GFAP aggregates were identified by their coarse appearance, bright fluorescence and apparently random distribution. The number of GFAP aggregates and their average size were quantified with the “Analyze particles” plug-in of ImageJ. Filament fragmentation was distinguished by the loss of continuity of filaments, which appeared as aligned particles. Percentages of cells bearing filaments, fragmented filaments or aggregates were determined by visual inspection of the preparations under the fluorescence microscope or from the acquired images.

Mitochondria were visualized by immunofluorescence with anti-TOM20. Mitochondrial length was assessed from 10 randomly selected mitochondria per cell from at least 20 cells. Alternatively, the proportion of cells displaying mitochondria longer than 2 μm was calculated. Colocalization of GFAP and mitochondria was evaluated by calculation of the Pearson and Manders coefficients using the JnCoP’s plug-in from ImageJ.

**Cell lysis and western blot** - Cell dishes (60 mm) were washed twice with cold PBS before addition of the lysis buffer (120 μl) containing 50 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% (v/v) SDS, 1 mM 2-mercaptoethanol, 50 mM sodium fluoride, 0.1 mM orthovanadate, 2 μg/ml each of protease inhibitors (leupeptin, aproatin and trypsin inhibitor) and 1.3 mM Pefabloc. For protein analysis under non-reducing conditions, 2-mercaptoethanol was omitted from the lysis buffer. Homogenization was carried out on ice by forced passes through a 26 1/2-G needle and the solution clarified by centrifugation at 10000 × g for 5 min at 6 °C. The protein concentration of the lysate was measured using the bicinchoninic acid (BCA) Protein assay kit (Pierce).

Aliquots of the lysates (20–30 μg) were denatured in Laemmli buffer in the presence (reducing conditions) or absence (non-reducing conditions) of 5% (v/v) 2-mercaptoethanol. Proteins were then separated on 10% SDS-PAGE gels and electrotransferred to Immobilon-P membranes (Millipore) that were subjected to a blocking step with 2% (v/v) low fat milk in Tween-Tris buffer saline (T-TBS, 20 mM Tris/HCl pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween-20), before incubation with the primary antibodies, at 1:500 or 1:1000 dilution in 1% (w/v) BSA in T-TBS, followed by three washes in T-TBS and the corresponding HRP-conjugated secondary antibodies at 1:2000 dilution in 1% (w/v) BSA in T-TBS. Incubations were typically carried out for 1 h at r.t. Signals were developed with the Enhanced Chemiluminiscence (ECL) system of GE Healthcare, detected on Agfa X-ray films and quantified using Scion Image.

**Assessment of cysteine accessibility and protein lipoxidation with biotinylated reagents** - For assessment of cysteine accessibility, U-87 MG cells transfected with either GFP-GFAP wt or R239C were treated with vehicle or 1 mM diamide for 15 min, lysed under non-reducing conditions as described above and free cysteine residues alkylated by incubating lysates (150 μg of total protein) with 2 mM biotinylated iodoacetamide (iab-C) for 30 min in the dark.

For evaluation of the incorporation of 15-PGJ2-B into cellular proteins, U-87 MG cells transiently transfected with GFP-GFAP wt or R239C were treated with vehicle or 7.5 μM 15d-PGJ2-B for 2 h and lysed as above. In parallel, conditioned media of the biotinylated reagents (150 μg of total protein) were then diluted to lower the SDS-PAGE bands below 0.1% (w/v) using 20 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1% (v/v) NP-40 and incubated with GFP-Trap beads (Chromotek) for immunoprecipitation. Proteins were loaded on SDS-PAGE gels and electrotransferred, and incorporation of iab-C or 15-PGJ2-B into proteins was assessed by detection of the biotin signal on blots with HRP-Streptavidin (GE Healthcare) and ECL. The total GFP-GFAP content was estimated by incubation with anti-GFP antibody.
The ratios lac-B/GFP and 15-PGJ2-B/GFP were calculated from scanning of the corresponding bands using Scion Image.

**Flow cytometry measurements of cellular redox status and mitochondrial function** - For assessment of basal redox status, U-87 MG cells stably transfected with GFP-GFAP wt or R239C cells were incubated for 30 min with 5 μM CellRox Deep Red (for detection of cytoplasmic reactive oxygen species (ROS)), 5 μM MitoSox Red (for detection of mitochondrial O2−) or 1 μM ThioTracker Violet (for detection of intracellular thiols). ThioTracker Violet incubations were performed in PBS supplemented with 0.49 mM MgCl2, 0.9 mM CaCl2, 5.5 mM glucose and 0.32 mM sodium pyruvate. For evaluation of the effects of 15d-PGJ2, cells were treated with either vehicle or 10 μM 15d-PGJ2 for 2 h at 37 °C in serum-free medium, and the fluorescent probes MitoSox Red (5 μM), CellRox (5 μM), TMRM (1 nM) or MitoTracker Deep Red (2 nM) were added for the last 30 min of the incubation. Afterwards, cells were trypsinized and centrifuged 5 min at 106 g at r.t., resuspended in cold PBS to achieve a density of 106 cells/ml, transferred to cytometer tubes and analyzed on CytoFLEX (for CellRox, ThioTracker Violet, TMRM and MitoTracker Deep Red assays) or FC500 (for MitoSox assays) flow cytometers. Cells bearing green fluorescence from GFP-GFAP wt or R239C were selected. A minimum of 104 events per condition were analyzed.

**Statistical analysis** - Experiments were performed at least three times with similar results. Statistical analysis was carried out using GraphPad Prism 5, and results are presented as mean values ± standard error of the mean (SEM). Statistical differences between selected pairs of data sets were evaluated using the unpaired two-tailed Student’s t-test and differences were considered significant when p < 0.5. When indicated, the One-way analysis of variance (ANOVA) for comparison of multiple data sets, followed by Tukey post-test, was performed. Significant differences are indicated by asterisks in the figures and the significance levels stated in the corresponding figure legend.

3. Results

**Expression of the AxD mutant GFP-GFAP R239C in astrocytic cells results in aberrant filament network organization** - We have previously shown that the single cysteine residue of GFAP, C294, is a key target for oxidative and lipoxidative modifications that disrupt the filament network [24]. The most relevant AxD mutations introduce new nucleophilic residues in the GFAP molecule (Fig. 1A), putatively increasing the susceptibility of the protein to lipoxidation. To address this possibility, we set out to explore the behavior of the more common and severe AxD mutation, the R239C substitution, in an astrocytoma cellular model under control conditions and under oxidative or electrophilic stress. The U-87 MG astrocytoma cell line expresses negligible levels of endogenous GFAP, compared to the U-251 MG astrocytoma cell line, as detected both by immunofluorescence (Fig. 1B) and western blot (Fig. 1C) using anti-GFAP polyclonal antibodies. Nevertheless, U-87 MG cells express similar vimentin levels than U-251 MG cells (Fig. 1C), which would support the formation of the intermediate filament network. Transient transfection of GFP-GFAP wt or the R239C mutant in U-87 MG cells led to the expression of high levels of the protein constructs, as detected by western blot. In addition to the bands corresponding to the protein monomers, bands of higher and lower molecular weight, likely representing oligomeric or partially degraded forms, respectively, were detected by the anti-GFAP antibody (Fig. 1C). Assessment of the distribution of the constructs 48 h after transient transfection by fluorescence microscopy (Fig. 1D, upper panels), revealed the distribution of GFP-GFAP wt mostly in extended filaments, although a low percentage of the cell population (~20%) showed a defective distribution with accumulations or aggregates (Fig. 1D, histogram). Remarkably, defects in network formation/extension were much more frequent in cells transfected with GFP-GFAP R239C, and were evident as aggregates and intense bundles in 70% of transfected cells (Fig. 1D, histogram). The differences in GFAP wt and R239C assembly were more obvious after stable transfection, when levels of the transfected proteins were more moderate (Fig. 1D, lower panels). Under these conditions, GFP-GFAP wt formed a homogeneous extended network in virtually all cells (Fig. 1D). In sharp contrast, the distribution of GFP-GFAP R239C did not improve under these conditions and aggregates of the protein were still present in approximately 80% of the cells (Fig. 1D, histogram). Notably, the extension of the GFAP network, as quantitated by the proportion of the cell area with detectable filaments, was also defective in cells expressing GFP-GFAP R239C, and was reduced by approximately 50% compared to the extension of GFP-GFAP wt detectable filaments (see below in Fig. 3B). Notably, transfection of U-87 MG cells with untagged GFAP constructs, namely, RFP/GFP wt or R239C, resulted in phenotypes similar to those of GFP-GFAP transfected cells (Fig. 1E), i.e., GFAP wt assembled in homogeneous extended filament networks whereas GFAP R239C formed thick bundles and aggregates, together with areas of more diffuse GFAP, as revealed by immunofluorescence.

AxD causing GFAP mutations are heterozygous. Nevertheless, as GFAP mutants seem to act in a dominant gain of function manner, the performance of the intermediate filament network would be globally affected. Although the precise balance of GFAP proteoforms in astrocytes is not known, a recent study analyzing diverse brain samples from AxD patients through gel electrophoresis and mass spectrometry has estimated that the proportion of mutant GFAP with respect to the wt form ranges from 21% to 46%, depending on the mutant/sample analyzed [37]. In order to simulate this situation, we transfected GFP-GFAP wt or R239C in U-251 MG astrocytoma cells, which express endogenous GFAP (Fig. 1F). GFP-GFAP wt uniformly incorporated into the endogenous GFAP network in a majority of cells, whereas GFP-GFAP R239C incorporation often disrupted the endogenous network leading to the formation of bundles and aggregates. Additionally, we explored the effects of GFP-GFAP wt or R239C transfection in rat primary astrocytes (Fig. 1G). Consistent with the observations in astrocytoma cells, GFP-GFAP wt displayed a homogeneous filamentous distribution while GFP-GFAP R239C formed more heterogeneous networks with coexistence of filaments, diffuse bundles and aggregates.

Taken together, these results show that expression of GFAP R239C by several transfection strategies leads to the formation of altered filament networks in cells types expressing different levels of endogenous GFAP. Nevertheless, U-87 MG cells stably transfected with GFP-GFAP constructs exhibited less variability in GFP-GFAP expression levels and network morphology, for which they were used for most of the subsequent studies.

The AxD mutant GFP-GFAP R239C is present in a more oxidized state in cells - GFAP is a target for lipoxidation and protein carbonyl formation in neurodegenerative diseases [20,23,38,39]. In order to
characterize the status of GFP-GFAP wt and R239C in the U-87 MG cellular model, we first explored the accessibility of their cysteine residues, as estimated by the incorporation of biotinylated iodoacetamide (Iac-B) (Fig. 2A). The extent of modification, detected with HRP-streptavidin, indicated slightly higher incorporation of Iac-B in GFP-GFAP R239C than in the wt (Fig. 2A) under basal conditions. However, this incorporation was in fact proportionally lower in the GFAP R239C mutant, which contains 2 cysteine residues, than in GFAP wt, which contains 1 cysteine residue (Iac-B incorporation of 0.6 versus 0.9 arbitrary units/cysteine residue in GFAP R239C and GFAP wt, respectively). Moreover, accessibility of GFP-GFAP R239C cysteine residues was decreased even further by treatment of cells with the oxidant diamide (Figs. 2A and 0.4 arbitrary units/cysteine residue), whereas that of GFAP wt was not significantly diminished. The relatively lower accessibility of GFAP R239C cysteine residues seemed selective since the overall incorporation of Iac-B into total lysate proteins, or in the band comigrating with actin, was not significantly different in cells expressing GFP-GFAP wt and R239C, either in the absence or presence of diamide (Fig. 2B–D). The lower accessibility of cysteine residues in GFP-GFAP R239C could be due to an alteration of the protein structure by the presence of the second cysteine residue leading to changes in the exposure of cysteine residues and/or to increased cysteine oxidation. Consistent with the latter possibility, electrophoresis under non-reducing conditions showed an increase in the presence of oligomeric species in GFP-GFAP R239C compared to GFP-GFAP wt and R239C constructs. Cells were transfected with RFP//GFAP wt or R239C and the distribution of GFAP was assessed by immunofluorescence 48 h after transfection. Insert, enlarged area showing aggregates, marked by arrows. (F) Expression of GFP-GFAP wt and R239C in U-251 MG cells transiently transfected with untagged GFAP wt or R239C constructs. Cells were transfected with RFP//GFAP wt or R239C and the distribution of GFAP was assessed by immunofluorescence 48 h after transfection. Insert, enlarged area showing aggregates, marked by arrows. (G) Rat primary astrocytes were transiently transfected with the indicated plasmids and the distribution of GFP-GFAP wt and R239C was monitored as above. Images shown are overall projections. In selected images, the approximate cell contour is highlighted by dotted lines. Bars, 20 μm.
intermediate filament proteins in SDS-PAGE gels, as previously characterized by us [24]. Nevertheless, the occurrence of heterooligomers of GFAP and other cellular proteins cannot be discarded from this type of analysis. Importantly, levels of GFAP oligomers were markedly lower under reducing conditions, indicating that they are formed at least in part by disulfide-bonded species. Curiously, the levels of GFP-GFAP wt or R239C oligomers did not increase upon treatment of cells with diamide (Fig. 2E). Interestingly, virtually identical results were obtained when cells were treated with H$_2$O$_2$ (Fig. 2F). Taken together, these results suggest that GFP-GFAP R239C is in a more "oxidized" state than GFP-GFAP wt in cells, due in part to disulfide-mediated oligomerization. In addition, the observation that exposure to diamide decreases cysteine

![Fig. 2. Accessibility of cysteine residues in GFP-GFAP wt and R239C transiently expressed in U-87 MG astrocytoma cells.](image-url)

- **A.** Total lysates + Iac-B analysis under control conditions and after treatment with 1 mM diamide for 15 min, evaluated by labeling proteins present in total cell lysates with biotinylated iodoacetamide (Iac-B). Total cell lysates and the fraction bound to a GFP-Trap resin were analyzed by SDS-PAGE. Iac-B incorporation into the GFP-GFAP band (arrowhead) was estimated from the biotin signal on blots, detected with HRP-streptavidin. Levels of GFP-GFAP constructs were assessed by western blot with an anti-GFP antibody. The histogram on the right shows results (mean ± SEM from three independent experiments) of the biotin signal of the GFP-Trap bound fusion constructs corrected by GFP levels. *p < 0.05 by unpaired Student’s t-test. The position of the cysteines on GFAP wt and R239C is schematized above the histogram. (B–D) Analysis of cysteine accessibility in total lysates of astrocytoma cells. Total cell lysates from U-87 MG cells transfected and treated as above were analyzed by gel electrophoresis. The biotin signal was detected with HRP-Streptavidin. The position of actin is indicated by the arrow. The total biotin signal in lysates (C) and the biotin signal associated with the actin band (D), corrected in both cases by actin levels assessed by western blot are presented as mean values ± SEM from three independent assays. (E and F) Effect of oxidants on the formation of GFP-GFAP wt and R239C oligomers. U-87 MG cells, transfected as above with GFP-GFAP wt or R239C, were treated with vehicle or with 1 mM diamide for 15 min (E) or 1 mM H$_2$O$_2$ for 1 h (F). Formation of GFAP oligomers was analyzed by SDS-PAGE of cell lysates under non-reducing and reducing conditions, followed by western blot with anti-GFAP. The position of the GFAP oligomers is indicated by an arrow. Results are representative of at least three independent experiments. The position of the molecular weight standards is indicated on the left of each image.
accessibility to Iac-B without increasing disulfide-bonded oligomers, strongly suggests the occurrence of other cysteine modifications, which could not be identified with this approach.

The R239C AxD mutation confers increased susceptibility to network disruption and toxicity induced by cell exposure to oxidants - The cellular distribution of GFAP is highly sensitive to modifications of its cysteine residue [24]. Therefore, we next explored the functional counterpart of the results described above by assessing GFAP reorganization in the presence of oxidants by fluorescence microscopy.

Treatment of U-87 MG cells stably transfected with GFP-GFAP constructs with diamide elicited a time-dependent fragmentation of GFAP filaments into particles or dots, which was significantly more severe in astrocytes expressing GFP-GFAP R239C (Fig. 3A).

Fig. 3. Effects of oxidants on the distribution of GFP-GFAP wt and R239C in stably transfected U87-MG cells. (A) Cells were treated with vehicle or 1 mM diamide for different time periods and the morphology of GFAP filaments was observed by confocal microscopy. Total projections are shown. Selected areas delimited by dotted squares appear enlarged in the insets, illustrating filament fragmentation upon diamide treatment. The histogram on the right depicts quantification of the percentage of cells exhibiting filament fragmentation under the indicated conditions. A total of at least 100 cells per experimental condition from three independent experiments were monitored. (B) Cells were treated with vehicle or 1 mM H$_2$O$_2$ for the indicated times and the distribution of GFP-GFAP was examined as above. Cell contours are indicated with dashed lines. Quantification of the area occupied by GFP-GFAP vs the total cell area from at least 30 cells per experimental condition is shown in the histogram. (C–E) The formation of aggregates in GFP-GFAP R239C expressing cells subjected to H$_2$O$_2$ treatment was characterized by additional quantifications, including the number of aggregates per cell (C), the total area occupied by aggregates (D) and the average size of aggregates (E). In all cases results are presented as average values ± SEM from at least three independent experiments. In (A) *p < 0.05, ***p < 0.0001 by unpaired Student’s t-test. In (B–D), **p < 0.001, ***p < 0.0001 by One-way Anova with Tukey post-test. Scale bars, 20 μm.
To confirm the vulnerability of GFP-GFAP R239C to oxidative damage, cells were subjected to a bolus treatment of H$_2$O$_2$ [40]. Exposure to H$_2$O$_2$ elicited relatively mild and reversible alterations in GFP-GFAP wt stably transfected cells, consisting in the condensation of the network at the proximity of the nucleus (Fig. 3B). After 30 min of treatment, the area occupied by the GFP-GFAP wt network with respect to the total cell area decreased about 50%, an effect that was reverted to a normal extended network after 2–4 h (Fig. 3B, graph). In contrast, astrocytoma cells stably expressing GFP-GFAP R239C showed a deficient extension of the filament network under basal conditions, which was even worsened by exposure to H$_2$O$_2$. Importantly, no GFP-GFAP R239C expressing cells could be detected after 4 h, suggesting irreversible damage and cell death. On the other hand, H$_2$O$_2$ treatment elicited the formation of coarse aggregates visible on confocal microscopy images, which occurred selectively in GFP-GFAP R239C-expressing cells. The number of these aggregates per cell increased markedly in the first 30 min of treatment, stabilizing thereafter (Fig. 3D), whereas the average size of the particles increased during the time course up to 4 h (Fig. 3E), maximal time for detection of GFP-GFAP R239C expressing cells. Therefore, these results indicate that organization of the GFP-GFAP R239C mutant is more susceptible to disruption by oxidants and suggest that cells expressing this protein form are more vulnerable to oxidative insults than GFP-GFAP wt.

**Effect of HNE on the distribution of GFAP wt and R239C** - The reactive aldehyde HNE has been involved in neurodegeneration [41, 42]. Moreover, GFAP-HNE adducts have been identified in brain tissue from Down’s syndrome patients [19]. Nevertheless, the precise effect of HNE on GFAP network distribution has not been characterized. Treatment with HNE induced a marked concentration-dependent juxtanuclear condensation of GFP-GFAP wt filaments in stably transfected U-87 MG cells. This condensation implied decreases of the cell area occupied by H$_2$O$_2$. Importantly, no GFP-GFAP R239C expressing cells could be detected after 4 h, suggesting irreversible damage and cell death. On the other hand, H$_2$O$_2$ treatment elicited the formation of coarse aggregates visible on confocal microscopy images, which occurred selectively in GFP-GFAP R239C-expressing cells. The number of these aggregates per cell increased markedly in the first 30 min of treatment, stabilizing thereafter (Fig. 3D), whereas the average size of the particles increased during the time course up to 4 h (Fig. 3E), maximal time for detection of GFP-GFAP R239C expressing cells. Therefore, these results indicate that organization of the GFP-GFAP R239C mutant is more susceptible to disruption by oxidants and suggests that cells expressing this protein form are more vulnerable to oxidative insults than GFP-GFAP wt.

**Fig. 4.** Effect of HNE on the distribution of GFP-GFAP wt and R239C in U-87 MG astrocytoma cells. (A) U-87 MG cells stably transfected with GFP-GFAP wt or R239C were treated with the indicated concentrations of HNE for 4 h and the organization of the GFP-GFAP network was assessed by confocal microscopy. Overall projections are shown and cell contours are indicated by dashed lines. Scale bars, 20 μm. The histogram at the right shows quantification of the changes in the area occupied by GFAP vs the cell area. (B-D) Characterization of the aggregates induced by HNE in GFP-GFAP R239C expressing cells showing the number of aggregates per cell (B), their total area (C), and their average size (D). In all cases, results are average values ± SEM from three independent experiments. At least 30 cells were analyzed per experimental condition. **p < 0.0001, **p < 0.01, *p < 0.05 by One-way ANOVA with Tukey post-test.
Importantly, these alterations particularly affected GFP-GFAP R239C expressing cells, in which treatment with 10 μM 15d-PGJ₂ elicited a more intense reduction of GFAP area than in those expressing the wt (Fig. 5A, graph). In all cases, at least 20 cells were analyzed per experimental condition. Results are presented as mean values ± SEM from three independent experiments. **p < 0.01, *p < 0.05 by One-way ANOVA with Tukey post-test. (E) U-87 MG astrocytoma cells transiently transfected with GFP-GFAP wt or R239C were incubated with 15d-PGJ₂-B and lipoxidation of the fusion proteins was assessed by immunoprecipitation with GFP-Trap and detection of incorporated biotin with HRP-Streptavidin (arrowhead). The histogram shows quantification of the biotin signal associated with the GFP-GFAP constructs retained in the beads (Bound) corrected by the levels of GFP detected by western blot (lower panel). TL, total lysates; FT, flow-through; W3, third wash of the beads. Results are shown as mean values ± SEM from at least three independent experiments. *p < 0.05 by unpaired Student’s t-test.

Fig. 5. Increased susceptibility of GFP-GFAP R239C expressed in U-87 MG astrocytoma cells to disruption and lipoxidation by 15d-PGJ₂. (A) Total projections of cells stably expressing GFP-GFAP wt or R239C treated with vehicle or the indicated concentrations of 15d-PGJ₂. Cell contours are outlined. Scale bars, 20 μm. The histogram on the right shows the quantification of the condensation of the GFP-GFAP network as the ratio between the area occupied by GFP-GFAP fluorescence and the total cell area. (B–D) Additional characterization of 15d-PGJ₂-induced morphological alterations. (B) Ratio of GFP-GFAP fluorescence at the perinuclear and at the submembranous regions. (C) Number and (D) total area of the aggregates observed in GFP-GFAP R239C expressing cells upon treatment with 15d-PGJ₂. In all cases, at least 20 cells were analyzed per experimental condition. Results are presented as mean values ± SEM from three independent experiments. **p < 0.01, *p < 0.05 by One-way ANOVA with Tukey post-test. (E) U-87 MG astrocytoma cells transiently transfected with GFP-GFAP wt or R239C were incubated with 15d-PGJ₂-B and lipoxidation of the fusion proteins was assessed by immunoprecipitation with GFP-Trap and detection of incorporated biotin with HRP-Streptavidin (arrowhead). The histogram shows quantification of the biotin signal associated with the GFP-GFAP constructs retained in the beads (Bound) corrected by the levels of GFP detected by western blot (lower panel). TL, total lysates; FT, flow-through; W3, third wash of the beads. Results are shown as mean values ± SEM from at least three independent experiments. *p < 0.05 by unpaired Student’s t-test.

Interestingly, formation of cyPG-protein adducts can be directly monitored by the use of cyPG biotinylated analogs. Incubation of U-87
MG cells transiently transfected with GFP-GFAP wt or R239C with biotinylated 15d-PGJ$_2$ (15d-PGJ$_2$-B) led to the incorporation of the cyPG in multiple polypeptides, as detected by gel electrophoresis followed by blot of total cell lysates and detection with HRP-streptavidin (Fig. 5E). Importantly, immunoprecipitation of the GFp-fusion proteins with GFp-trap clearly showed a nearly 2-fold increase in the intensity of the biotin signal associated with GFP-GFAP R239C with respect to GFP-GFAP wt, indicating that the presence of a second cysteine residue in the mutant leads to increased lipoxidation by 15d-PGJ$_2$-B. Taken together, these observations demonstrate that the GFp-GFAP R239C mutant undergoes increased lipoxidation and more severe disruption than GFp wt when exposed to 15d-PGJ$_2$.

**Influence of GFAP R239C in the cellular redox status** - The results shown above indicate that GFAP R239C is more susceptible than the wt protein to oxidative damage and lipoxidation-induced disruption of the network. Nevertheless, given the fact that GFP-GFAP R239C appears to be more oxidized under basal conditions, the possibility exists that expression of this protein influences the redox status of cells towards a more oxidative environment. In order to address this aspect, several parameters were measured by flow cytometry in untreated U-87 MG cells stably transfected with GFP-GFAP wt or R239C (Fig. 6). Assessment of cytoplasmic ROS using CellRox did not evidence significant differences between cells expressing GFP-GFAP wt or R239C (Fig. 6A). In contrast, mitochondrial superoxide production, detected with MitoSox, was significantly higher in cells expressing GFP-GFAP R239C under basal conditions (Fig. 6B). Moreover, estimation of intracellular free thiols with ThioloTracker, which is assumed to reflect mainly GSH thiol [46], revealed a clear decrease in free thiol content (~30%) in cells expressing the AxD mutant (Fig. 6C). Therefore, according to these observations, just the expression of GFP-GFAP R239C results in cellular oxidative stress.

Expression of the AxD GFAP R239C mutant elicits mitochondrial alterations in astrocytoma cells - Mitochondria are key sources and targets of ROS. The results shown above suggest a putative damage of mitochondria in GFP-GFAP R239C expressing cells; therefore, we analyzed the localization and morphology of these organelles in our cellular model by immunostaining of TOM20, a translocase of the outer mitochondrial membrane (Fig. 7A). In U-87 MG astrocytoma cells stably expressing GFP-GFAP wt, mitochondria appeared as dots or small elongated structures distributed throughout the cytoplasm. In contrast, in GFP-GFAP R239C expressing cells, mitochondria showed a tendency to accumulate near the nucleus and frequently appeared as long chains, spanning several microns, suggestive of defective mitochondrial fission. Overall mitochondrial length was found to be nearly 2-fold larger in GFP-GFAP R239C expressing cells. Moreover, nearly 80% of the mitochondria in cells expressing the AxD GFAP mutant were longer than 2 μm compared to 20% in cells expressing GFP-GFAP wt (Fig. 7A).

Type III intermediate filaments, including vimentin and desmin, have been shown to interact with and regulate mitochondria homeostasis [47, 48]. Interestingly, a certain degree of coincidence between GFAP and TOM20 signals was observed in both GFP-GFAP wt and R239C expressing cells (Fig. 7B, highlighted for enlarged selected regions in the right panels). In addition, GFAP-TOM20 colocalization was moderately, but significantly, higher in cells expressing the mutant, as indicated by calculation of the Pearson (0.68 ± 0.01 vs 0.72 ± 0.01) and Manders (M1, TOM20 overlapping GFAP: 0.65 ± 0.02 vs 0.79 ± 0.02) coefficients (see below in Fig. 7D and E).

Next, we subjected the cells to lipoxidative stress by treatment with 15d-PGJ$_2$. In cells expressing GFP-GFAP wt, 15d-PGJ$_2$ elicited the appearance of more interconnected mitochondria, which frequently arranged in circles adopting a net-like disposition (Fig. 7C). Remarkably, in GFP-GFAP R239C expressing cells, mitochondria were further elongated upon 15d-PGJ$_2$ treatment, and could reach over 6 μm of length. Moreover, treatment with 15d-PGJ$_2$ significantly increased the extent of GFAP-TOM20 colocalization, as quantitated by the Pearson coefficient, both in cells expressing GFAP wt and mutant (Fig. 7D).

Expression of GFP-GFAP R239C alters mitochondrial function - Potential effects on mitochondrial function associated with the
Fig. 7. Mitochondrial alterations induced by stable expression of GFP-GFAP wt or R239C in U-87 MG astrocytoma cells evaluated by confocal microscopy.

(A) Mitochondrial morphology was assessed by immunofluorescence with anti-TOM20 (red) of cells expressing the GFP-tagged GFAP proteins (green). Representative images of overall projections are shown for mitochondria (red) and merged channels (inset). Bars, 20 μm. The regions of interest of the red channel delimited by dashed squares are enlarged at the right. Bars, 10 μm. Histograms depict quantification of mitochondrial length (left) and percentage of long mitochondria (>2 μm, right) assessed from at least 20 cells per experimental condition. Results are presented as average values ± SEM of three independent experiments. ***p < 0.0001 by unpaired Student’s t-test.

(B) Details of the overlap between the GFP-GFAP wt or R239C networks (green) and mitochondria stained with TOM20 (red). Images are single confocal sections acquired with the Leica SP8 Lightning module for improved resolution. Individual channels and merged images are shown. Areas of interest delimited by dashed squares are enlarged at the right, and pseudocolor images highlighting areas of coincidence in gray scale are provided.

(C) U-87 MG cells stably expressing GFP-GFAP wt or R239C were treated with vehicle or 10 μM 15d-PGJ2 for 2 h, and mitochondria were stained with TOM20. Overall projections of merged channels are shown at the left. Cell contours are outlined. Areas delimited by dashed squares are enlarged at the right showing morphological details of mitochondria, where white arrows indicate interconnected mitochondria. Scale bars, 20 μm. (D,E) Histograms in the lower panels show the Pearson (D) and the Manders’ overlap coefficient (E), calculated as the TOM20 signal overlapping the GFP-GFAP signal. At least 30 cells were quantified per experimental condition. Results are presented as mean values ± SEM from three independent experiments. ***p < 0.0001, **p < 0.01, *p < 0.05 by unpaired Student’s t-test.
morphological changes induced by expression of the GFP-GFAP R239C mutant were assessed by flow cytometry using MitoTracker Deep Red to measure mitochondrial mass and tetramethylrhodamine (TMRM) to analyze the mitochondrial membrane potential. Under basal conditions, mitochondrial mass was decreased by nearly 30% in GFP-GFAP R239C vs wt expressing cells (Fig. 8A), which may indicate a diminution in the number of mitochondria. Non-significant changes in mitochondrial mass were detected upon 15d-PGJ2 treatment of cells expressing GFP-GFAP wt or R239C, although a trend towards a decreased mass was still observed in cells bearing the wt protein (Fig. 8A).

Analysis of membrane potential did not show significant differences between GFP-GFAP wt and R239C expressing cells under basal conditions (Fig. 8B). Remarkably, treatment with 15d-PGJ2 elicited a moderate hyperpolarization of mitochondria (of approximately 25%), which was statistically significant in cells expressing GFAP R239C (Fig. 8B).

Given the important role of mitochondria in the generation of ROS, we next explored the production of superoxide anion (O2-) with the MitoSox Deep Red probe, which directly targets this organelle. Consistent with the results shown in Fig. 6B, cells expressing GFP-GFAP R239C indeed produced higher levels of mitochondrial superoxide compared to cells expressing GFP-GFAP wt (Fig. 8C). Additionally, a challenge with 15d-PGJ2 elicited a nearly 2-fold increase in superoxide production in GFP-GFAP wt expressing cells. Moreover, 15d-PGJ2 significantly increased the already high mitochondrial superoxide generation of cells bearing GFP-GFAP R239C (Fig. 8C), although the extent of the
enhancement (nearly 1.5-fold) was more modest than in cells bearing GFAP wt. Taken together, these results clearly demonstrate an altered mitochondrial function in cells expressing the AxD mutant GFP-GFAP R239C, with increased superoxide generation, which, in turn could contribute to oxidative stress.

Behavior of the AxD mutants GFAP R239H and R239G. Besides the substitution by cysteine, other pathogenic mutations at the R239 site have been reported, namely, R239H, R239L, R239P and R239G (https://alexander-disease.waisman.wisc.edu/). Therefore, we explored the characteristics of two additional mutants, R239H and R239G. Transient transfection of GFP-GFAP into U-87 MG cells led to an abnormal distribution of the protein in a high proportion of cells, characterized by

Fig. 9. Impact of the expression of GFP-GFAP R239G or R239H in astrocytoma cells. (A) The morphology of the intermediate filament network was assessed by fluorescence microscopy of U-87 MG astrocytoma cells transiently transfected with the indicated plasmids. The graph depicts the proportion of cells displaying aggregates. Results are average values ± SEM from three experiments. A minimum of 40 cells were monitored per experimental condition. *p < 0.05 by unpaired Student’s t-test. (B) Susceptibility of the GFP-GFAP wt, R239G or R239H network to disruption by treatment with vehicle (DMSO, control) or 10 μM HNE for 4 h was assessed in cells transfected as above. Graph depicts the proportion of cells bearing aggregates under the various experimental conditions as average values ± SEM of three experiments. **p < 0.01, *p < 0.05 by unpaired Student’s t-test. At least 100 cells were monitored per experimental condition. Bars, 20 μm. (C) Mitochondrial morphology was evaluated in cells transfected as in (A) by immunofluorescence with TOM20 antibody. Representative images of overall projections are shown for mitochondria (red) and merged channels (insets). The regions of interest of the red channel delimited by dashed squares are enlarged at the right (bars, 10 μm). Graph depicts the proportion of cells harboring mitochondria longer than 2 μm. Results are average values ± SEM from three experiments totaling 128 cells. **p < 0.01 by unpaired Student’s t-test.
filament condensation in bundles and frequent appearance of aggregates, even in cells expressing low levels of the construct (Fig. 9A). These features were clearly reminiscent from those of GFP-GFAP R239C. In contrast, GFP-GFAP R239G distributed uniformly in most cells, displaying a pattern more similar to GFP-GFAP wt, with a moderate proportion of cells showing aggregates (Fig. 9A). We next explored the susceptibility of these mutants to disruption by lipoxidation upon HNE treatment in comparison with GFP-GFAP wt (Fig. 9B). Similar to the results presented in Fig. 4, treatment of cells transiently transfected with GFP-GFAP wt with HNE led to bundle condensation without increasing the proportion of cells with aggregates (Fig. 9B). In contrast, HNE treatment increased the proportion of GFP-GFAP R239G or R239H expressing cells that displayed aggregates, indicating that they are more susceptible to HNE effects. Finally, evaluation of mitochondrial morphology revealed that a higher proportion of the cells transfected with GFP-GFAP R239H contained mitochondria longer than 2 μm, compared to those transfected with GFP-GFAP R239G (Fig. 9C). Taken together, these observations suggest that in this cellular model, there is a different tolerance to mutations at the GFAP 239 site, with substitutions by a small residue such as glycine being better tolerated than changes to amino acids with a bulkier lateral chain, such as histidine, or a strongly nucleophilic residue, such as cysteine. Nevertheless, our results suggest that the various GFAP R239 mutants tend to diminish the capability of cells to cope with oxidative and/or electrophilic stress.

4. Discussion

AxD is a neurodegenerative disease that begins in astrocytes and causes a profound derangement of brain homeostasis. Although the cause of AxD is well known to reside in mutations in the GFAP sequence, the disease pathogenesis is still not fully understood. Here we have taken a streamlined approach to analyze the cellular consequences of GFAP mutation by expressing GFP-GFAP wt and AxD mutants in astrocytoma cells. Our results show that expression of the AxD R239C mutant protein in this cell model is sufficient to elicit oxidative stress and disrupt the morphology and function of mitochondria. As in AxD, this mutant GFAP displays alterations in assembly and forms aggregates. Importantly, GFP-GFAP R239C shows higher basal oxidation and increased susceptibility to lipoxidation by 15d-PGJ2 and to disruption by oxidants and electrophilic lipids, such as HNE. Moreover, this higher susceptibility is translated into cytotoxicity, as observed after H2O2 challenge. Taken together, our results suggest that oxidative stress, mitochondrial dysfunction and protein (lip)oxidation may constitute a feedback loop for disease progression in certain forms of AxD. A schematic view of these events is presented in Fig. 10. Nevertheless, distinct GFAP mutations could trigger not totally overlapping pathogenic mechanisms. Indeed, two other mutants at the 239 site, namely, GFP-GFAP R239G and R239H show dissimilar assembly defects and associated mitochondrial alterations, although both of them undergo increased aggregation upon treatment with HNE with respect to the wt.

Our work has mainly focused on the GFAP R239C mutant for two reasons: first, it constitutes one of the most frequent and deleterious mutations affecting AxD patients [1], and second, it presents an additional cysteine residue which could make the mutant protein more susceptible to oxidative or lipoxidative damage. By analogy with vimentin [49], R239 could be oriented towards the space between the two monomers in the GFAP dimer. Therefore, the R239C substitution could disrupt inter- and intra-molecular interactions, such as hydrogen bonds, and/or introduce new repulsion forces or steric hindrances. Since the rod domain of GFAP is essential for the initial steps of dimerization [50], these disturbances could destabilize GFAP filaments, decreasing their solubility and promoting aggregation, as suggested for the L123P or D128N AxD mutations [51]. However, the R239C mutant is able to elongate in vitro, forming the typical 10 nm filaments [52]. Therefore, additional factors should contribute to defects in network organization, among them, GFAP overexpression or alterations in protein-protein interactions. On the other hand, mutant residues could be targets for different posttranslational modifications, contributing to misfolding or network disruption.

Therefore, development of experimental models mimicking AxD is...
is more susceptible than the wt to disruption by oxidants. Further cells expressing GFAP wt. These observations indicate that GFAP R239C residues is further decreased and the fragmentation of the mutant oxidant diamide showed that the accessibility of GFAP R239C cysteine degree of oxidation could contribute to the anomalous organization of electrophiles resulting in network disruption [24]. Here we have network, likely due to the presence of low levels of the mutant protein combination of strategies can be valuable to get mechanistic insight and potentially develop therapeutic approaches.

In our cellular models, expression of GFP-GFAP R239C leads to clear alterations in GFAP network formation, with the appearance of cytoplastic aggregates that are reminiscent of Rosenthal fibers. Similarly, the R239H mutant, which introduces a bulky, although less nucleophilic residue at this position, also forms abundant aggregates and disrupts the intermediate filament network. In contrast, substitution of R239 by a glycine residue, which lacks a lateral chain and potentially confers higher flexibility to the polypeptide backbone, is better tolerated under control conditions. Interestingly, in all cases there is a small proportion of cells expressing GFAP mutants that display a nearly normal filament network, likely due to the presence of low levels of the mutant protein that are able to incorporate into the endogenous intermediate filament network without inducing disruption. Conversely, as previously reported [56], GFAP overexpression, even of the wild type protein, is poorly tolerated and may result in abnormal assembly. Indeed, it has been previously proposed that AxD pathogenic mechanisms involve an increase in GFAP expression that reaches a threshold above which aggregation occurs, with sequestration of chaperones, alterations in pro teaseomal degradation and activation of stress pathways (reviewed in Ref. [57]).

Cysteine residues are highly nucleophilic and prone to modification. Indeed, the single cysteine residue of GFAP wt, C294, is targeted by electrophiles resulting in network disruption [24]. Here we have observed that cysteine accessibility of GFP-GFAP R239C is proportionally lower than that of GFAP wt, which, together with the appearance of disulfide crosslinked oligomers of GFAP R239C under basal conditions, indicates that the mutant protein is more oxidized in cells. This higher degree of oxidation could contribute to the anomalous organization of GFP-GFAP R239C, including formation of aggregates and the retraction of the network from the cell periphery. Importantly, treatment with the oxidant diastere showed that the accessibility of GFAP R239C cysteine residues is further decreased and the fragmentation of the mutant network into small dots is more severe compared to that occurring in cells expressing GFAP wt. These observations indicate that GFAP R239C is more susceptible than the wt to disruption by oxidants. Further confirmation of this property was obtained by a bolus treatment with H2O2, which elicits reversible alterations in cells expressing GFP-GFAP wt, but irreversible toxicity and massive potentiation of aggregate formation in GFP-GFAP R239C expressing cells. Similarly, an increased susceptibility of cultured astrocytes from GFAP R239H transgenic mice to H2O2 toxicity has been previously reported, although this was not accompanied by increased formation of aggregates [58]. Taken together, these observations suggest an important role of the additional cysteine residue in the response of GFAP R239C to oxidants.

The higher susceptibility of GFP-GFAP R239C to electrophiles, HNE and 15d-PGJ2, is clearly demonstrated by the fact that disruption of the network organization occurs at lower concentrations of the reactive agents in cells expressing the AxD mutant than in those expressing GFP-GFAP wt. Lipoxidation of GFAP in neurodegeneration has been reported [19,23]. In cultured astrocytes we have confirmed that the single cysteine, C294, for GFP wt lipoxidation and for its reorganization upon treatment with cyPG [24]. Here we have confirmed that 15d-PGJ2 incorporation into GFP-GFAP R239C is 2-fold higher than that occurring in GFP-GFAP wt in astrocytoma cells. Moreover, 15d-PGJ2 potentiated GFP-GFAP R239C aggregation formation, thus suggesting an effect of lipoxidation on network disruption and aggregation. Interestingly, this cyPG has been previously reported to provoke protein aggregation through various mechanisms, including induction of protein crosslinking, either through direct bis-adduct formation [59,60], or secondary to oxidative stress, or through protein unfolding and coalescence, as reported for UCH-L1 [61]. Interestingly, lipoxidized proteins can also inhibit proteasome activity, and several components of the proteasomal degradation machinery are direct targets for modification and inhibition by electrophilic lipids, in turn contributing to proteostasis. Indeed, HNE additives have been found in Rosenthal fibers [27]. Overall, these data point towards the putative implication of these mechanisms in the accumulation and aggregation of lipoxidized pro teins, including GFAP.

Therefore, our results show that an exacerbation of aggregate formation is a common consequence of the exposure of GFP-GFAP R239C expressing cells to oxidants and electrophiles. Similarly, an increase in the proportion of cells bearing aggregates upon treatment with HNE is also observed in cells expressing GFP-GFAP R239H or R239G. Accumulating evidence indicates that there is a bidirectional crosstalk between protein oxidation and aggregation [62]. Protein oxidation is known to induce aggregation through various mechanisms including protein crosslinks or unfolding and formation of amyloid-like assemblies [63,64]. In turn, protein aggregation can induce ROS production [62,64], potentiating protein oxidation. Additionally, protein aggregation could directly favor oxidation, as suggested by the increased oxidation rates of certain protein residues found in crowded environments [65], thus potentially reinforcing a vicious cycle. Formation of intracellular aggregates is a hallmark of numerous neurodegenerative diseases, as illustrated by the aggregates of Aβ peptide in Alzheimer’s, α-synuclein in Parkinson’s or huntingtin in Huntington’s disease (reviewed in Ref. [66]), in which oxidative stress and generation of reactive species also occurs. Nevertheless, the pathogenic significance of the aggregates is not completely understood. Both a deleterious and a protective role of protein oxidation and aggregation have been proposed, given the higher toxicity of some protein oligomers. Nonetheless, it seems clear that the vicious cycle of protein oxidation and aggregation has harmful consequences including endoplasmic reticulum stress, impairment of protein degradation, and cell toxicity [62,64]. In our model system, aggregate formation becomes massive under certain conditions, correlating with irreversible cell damage.

Our results also indicate that just the expression of GFP-GFAP R239C is sufficient to induce oxidative stress in cells with respect to those expressing the wt protein, as shown by decreased free thiol content, putatively reflecting a drop in GSH levels, and by the increased generation of mitochondrial O2. Having in mind that the antioxidant defense of neurons mainly arises from the release of GSH from astrocytes [67] (reviewed in Ref. [68]), it is tempting to hypothesize that the fall in astrocyte GSH levels, observed in this work, could take place in AxD, in turn leading to a decline in neuronal GSH availability, with adverse consequences for the protection of neurons against oxidative damage. Given the limitations of cellular models, additional work is required to ascertain this hypothesis. The maintenance of cells under standard cell culture conditions, therefore in the presence of oxygen levels higher than those encountered in brain tissue [69], can result in a “basal oxidative stress”, which could be influenced as well by the expression of GFP fusion constructs [70]. Nevertheless, under these conditions, expression of GFP-GFAP R239C clearly exacerbated oxidative stress with respect to GFP-GFAP wt.

Importantly, under our experimental conditions, we observe deep alterations of mitochondria in GFP-GFAP R239C-expressing astrocytoma cells, including a decrease in mitochondrial mass and increased superoxide generation, and elongated appearance. Elongated mitochondria appear also in GFP-GFAP R239H, and to a lesser extent in GFP-GFAP R239G transfected cells. A rise in superoxide generation could contribute to oxidative stress and protein oxidation, thus feeding the damaging cycle of protein aggregation-oxidation. Moreover, oxidative stress could affect mitochondrial dynamics in various ways and either inhibit or stimulate mitochondrial fission, as it has been shown previously [71,72] (reviewed in Ref. [73]), thus contributing to the
altered morphology/elongated appearance of mitochondria. In some settings, inhibition of mitochondrial fission or induction of mitochondrial fusion have been viewed as defense mechanisms aimed to minimize mitochondrial damage [74,75]. Nevertheless, mitochondrial hyperfusion could impair the correct recycling of mitochondria through mitophagy (reviewed in Ref. [76]), therefore perpetuating mitochondrial dysfunction. The cyPG 15d-PGJ2 has been reported to target mitochondria eliciting various functional alterations, including formation of ROS [77] and transient decrease of mitochondrial membrane potential [78]. Here we observed that 15d-PGJ2 tended to decrease mitochondrial mass and stimulated mitochondrial superoxide production, both in GFP-GFAP wt and R239C-expressing cells. Interestingly, 15d-PGJ2 elicited the appearance of elongated mitochondria in GFAP wt, and of markedly long, hyperfused mitochondria in GFAP R239C-expressing cells. This effect could be related to the ability of 15d-PGJ2 to modify the mitochondrial fission protein DRP1, provoking its oligomerization and inhibition [79]. It should be noted that contrarily to what has been described in several models of neurodegenerative disease, our results do not show a loss of mitochondrial membrane potential in our cellular model. Instead, cells expressing GFAP wt or mutant display similar mitochondrial membrane potential under basal conditions, whereas slight increases occur in mutant cells treated with 15d-PGJ2, in association with increased ROS production and, potentially, fission occurrence.

Interestingly, we have observed a moderate colocalization of GFPPGFAP with the mitochondrial membrane translocase TOM20, which is slightly higher in cells expressing GFP-GFAP R239C. Interactions of type III intermediate filaments with mitochondria have been previously reported. The N-terminus of vimentin has been described to directly interact with mitochondria [48,80], in a process regulated by the GTPase Rac1 [81], which increases the mitochondrial membrane potential [48]. Besides, this interaction influences mitochondria positioning and motility [47], and protects these organelles from oxidative stress [81]. Mutations or knockout of other cytoskeletal proteins, such as desmin, plectin or tubulin have also been associated with mitochondrial dysfunction [82], thus highlighting the importance of the cytoskeleton in maintaining mitochondrial homeostasis. Although further work is needed to elucidate the nature of the interaction of GFAP with mitochondria and how GFAP R239C elicits mitochondrial dysfunction, it can be hypothesized that abnormal contacts established with the AxD mutant could contribute to alterations in mitochondrial motility or fission. Furthermore, astrocytic mitochondrial alterations could have functional consequences in neurons. Transfer of mitochondria from astrocytes to neurons appears to be essential for neuronal ATP synthesis, neuroprotection and neurorecovery after stroke [83]. Interestingly, alterations in mitochondrial transfer have been observed in cellular models of AxD [84]. It should be important to ascertain whether the presence of hyperfused mitochondria elicited by mutant GFAP, as observed in our model, has negative consequences for mitochondrial transfer.

Importantly, AxD can be associated with many different single point mutations in GFAP. Mutations in more than 90 of the 432 residues of GFAP have been reported, of which only five are considered non-pathogenic [7]. Among the pathogenic mutations, some cause infantile (onset before 2 years of age) and others adult-onset disease [7]. The impact of a given mutation on GFAP assembly and/or interactions may determine both the cellular alterations and the final clinical entity. Five variants have been reported at the R239 position, which in most cases associate with the infantile disease [85], although R239G has been reported in several late onset cases [86–88]. Interestingly, in our U-87 MG astrocytoma cell model, the GFP-GFAP R239G, R239H and R239C mutants alter filament organization and mitochondrial morphology to different extents. These observations could suggest a correlation between the extent of the cellular alterations induced by these mutants and the severity of the disease. Nevertheless, further studies are needed to confirm this hypothesis.

In summary, although the exact order of events cannot be ascertained, the results from our cellular model, together with previous evidence, suggest a pathogenic cycle, depicted in Fig. 10, involving an alteration of GFAP assembly, which could be initially mild but sufficient to induce mitochondrial dysfunction and oxidative stress. This could elicit GFAP oxidation leading to further misfolding and aggregation, in turn potentiating oxidative stress and the profound derangement of astrocyte functions. Alternatively, GFAP aggregation could be the initial event, although it would be further potentiated by oxidative stress and protein oxidation or lipoxidation. Our results pave the ground for detailed study of GFAP oxidative or electrophilic modifications in AxD, and their potential involvement in disease pathogenesis. Importantly, extra cysteine residues appear in a high proportion of the reported AxD cases. Therefore, the higher nucleophilicity of this residue and increased susceptibility of mutant GFAP to lipoxidation could constitute a pathogenic factor influencing many aspects of the disease.

Author contributions

AVP performed experiments, analyzed data and prepared illustrations; PGJ performed experiments; MAP participated in manuscript writing and organization and figure design; DPS designed and coordinated the study, wrote the manuscript and edited figures.

Funding

This work was supported by Agencia Estatal de Investigacion, MICINN/ERDF (grants SAF2015-68590R and RTI2018-097624-B-100), and ISCIII/ERDF (RETIC ARADYL RD16/0006/0021). Spain. AVP and PGJ were supported by FPI grants BES-2016-076965 and PRE2019-088194 from MICINN/ERDF, respectively (ERDF, co-funded by the European Regional Development Fund).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Feedback from EU COST Actions EuroCellNet (CA15214) and EpilipidNet (CA19105), and from the Alexander Consortium (EJP RD COFUND-EJP N° 825575) is gratefully acknowledged. We thank the personnel of the Optical Microscopy and Flow Cytometry facilities at Centro de Investigaciones Biológicas Margarita Salas for their advice. The technical assistance of MJ Carrasco at the early stages of this work is very much appreciated.

References

[1] R.A. Quinlan, M. Brenner, J.E. Goldman, A. Messing, GFAP and its role in Alexander disease, Exp. Cell Res. 313 (10) (2007) 2077–2087.
[2] E.M. Hol, M. Pekny, Gial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system, Curr. Opin. Cell Biol. 32 (2015) 121–130.
[3] A. Messing, Alexander disease, Handb. Clin. Neurol. 148 (2018) 693–700.
[4] A.B. Johnson, M. Brenner, Alexander’s disease: clinical, pathologic, and genetic features, J. Child Neurol. 18 (9) (2003) 625–632.
[5] C. Eliaison, C. Sahlgren, C.H. Berthold, J. Stakeberg, J.E. Celis, C. Bethelholtz, J. E. Eriksson, M. Pekny, Intermediate filament protein partnership in astrocytes, J. Biol. Chem. 274 (34) (1999) 23996–24006.
[6] J. Middeldorp, E.M. Hol, GFAP in health and disease, Prog. Neurobiol. 93 (3) (2011) 421–443.
[7] A. Messing, M. Brenner, GFAP at 50, ASN neuro 12 (2020), 1750091420949680.
Adaptive autophagy in Alexander disease-affected astrocytes, Autophagy 4 (5) (2008) 701–708.

V. Andrade, L.P. Navarrete, M.G. Pastor, A. Martinez, M. Portero-Otin, A. Naudi, R. Pamplona, I. Ferrer, Type-III intermediate filaments as a potential pathogenic mechanism, J. Neurochem. 123 (4) (2012) 622–634.

M. Pekny, M. Pekna, Astrocyte reactivity and reactive astrogliosis: costs and benefits, Physiol. Rev. 94 (4) (2014) 1077–1098.

M. Dominguez, E. de Oliveira, M.A. Odena, M. Portero, R. Pamplona, I. Ferrer, Protein targets of oxidative lipoxidative damage as a potential pathogenic mechanism, J. Neurochem. 123 (4) (2012) 622–634.

A. Martinez, M. Portero-Otin, R. Pamplona, I. Ferrer, Protein targets of oxidative damage in human neurodegenerative diseases with abnormal protein aggregates, Brain Res. 20 (2) (2010) 281–297.

H. Kosako, M. Amano, M. Yanagida, K. Tanabe, Y. Nishi, K. Kaibuchi, M. Inagaki, M. Pekny, M. Pekna, Astrocyte reactivity and reactive astrogliosis: costs and benefits, Physiol. Rev. 94 (4) (2014) 1077–1098.
