

Alexander disease – the road ahead

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Abbreviations: ALXDRD, Alexander disease; CNS, central nervous system; FRAP, fluorescence recovery after photobleaching; GFAP, glial fibrillary acidic protein; ULFs, unit length filaments; Vim, vimentin; wt, wild type.

ABSTRACT

Alexander disease is a rare neurodegenerative disorder caused by mutations in the glial fibrillary acidic protein (GFAP), a type III intermediate filament protein expressed in astrocytes. Both early (infantile or juvenile) and adult onsets of the disease are known and, in both cases astrocytes present characteristic aggregates, named Rosenthal fibers. Mutations are spread along the GFAP sequence causing disruption of the typical filament network in a dominant manner. Although the presence of aggregates suggests a proteostasis problem of the mutant forms, this behavior is also observed when expression of wild type GFAP is increased. Additionally, several isoforms of GFAP have been described to date, while the impact of the mutations on their expression and proportion has not been exhaustively studied. Moreover, the posttranslational modification patterns and/or the protein-protein interaction networks of the GFAP mutants may be altered, leading to functional changes that may modify the morphology, positioning, and/or the function of several organelles, in turn, impairing astrocyte normal function and subsequently affecting neurons. In particular, mitochondrial function, redox balance and susceptibility to oxidative stress may contribute to derangement of GFAP mutant-expressing astrocytes. In order to study the disease, and to develop putative therapeutic strategies, several experimental models have been developed, a collection that is in constant growth. The fact that most cases of Alexander disease can be related to GFAP mutations, together with the availability of new and more relevant experimental models, holds promise for the design and assay of novel therapeutic strategies.

1. Introduction

Protein aggregation, inflammation and oxidative stress are common hallmarks of neurodegenerative diseases of diverse etiology, both common and rare. Among the latter, Alexander disease (ALXDRD; OMIM 203450) is a fatal neurodegenerative disorder that often disrupts the white matter of the brain, and hence is classified as a leukodystrophy. Its clinical manifestations may include severe motor impairment and intellectual disability, epilepsy, megalencephaly and seizures (Messing, 2018a). While seizures and macrocephaly seem more common in infantile than in adult onset of ALXDRD, palatal myoclonus has been only described in the later form of the disease (Balbi et al., 2010; Brenner et al., 2009). The cause of ALXDRD are mostly *de novo* mutations in glial fibrillary acidic protein (GFAP), a type III intermediate filament protein expressed mainly in astrocytes, which are key players in brain function and homeostasis (Messing, 2018a; Pekny et al., 2016). However, some cases of inherited pathogenic mutations have been also reported (Li et al., 2006; Zang et al., 2013). The continuously increasing number of reported GFAP mutations associated with ALXDRD can be found in a dedicated database supported and updated by Messing's group (<https://alexander-disease.waisman.wisc.edu>).

ALXDRD mutations are mainly heterozygous single point substitutions, which can occur at diverse locations in the GFAP sequence. Remarkably, the mutant proteins display a dominant behavior disrupting the assembly of the intermediate filament network and eliciting the formation of characteristic protein aggregates, known as Rosenthal fibers, in the cytoplasm of astrocytes. Rosenthal fibers may contain not only GFAP, but also a variety of other proteins involved among others in stress response (e.g. α B-crystallin and Hsp27)(Der Perng et al., 2006; Iwaki et al., 1989), transcription (e.g. cJun)(Tang et al., 2006), and protein degradation (e.g. ubiquitin, proteasome 20S)(Lowe et al., 1988; Tang et al., 2010), together with other intermediate filament proteins and factors regulating their assembly (e.g. plectin, synemin, vimentin)(Heaven et al., 2016; Pekny et al., 2014; Tian et al., 2006). Either due to the impairment of supporting functions or toxic effects of astrocytes carrying mutant GFAP, intercellular communication with neurons, and putatively with oligodendrocytes forming the myelin around axons, and brain homeostasis are compromised in ALXDRD leading to neurodegeneration. In this line, nitric oxide release from astrocytes has been shown to trigger neurodegeneration in an experimental model of ALXDRD (Wang et al., 2015). Nevertheless, the molecular pathogenesis of ALXDRD remains largely unknown. Therefore, more detailed studies on specific GFAP mutants associated with different ALXDRD types/onsets are needed. Here we will summarize the current knowledge on several aspects of ALXDRD emphasizing those that, in our opinion, deserve a stronger research effort in the near future.

2. GFAP protein structure and regulation

The *GFAP* gene encodes the canonical GFAP α form spanning 432 residues, as well as several alternative spliced isoforms (Hol and Pekny, 2015; van Asperen et al., 2022), which differ mainly in their C-terminal end. GFAP consists of structurally disordered head and tail domains and a central coiled-coil rod domain containing the single cysteine residue (C294) of the protein. Assembly of type III intermediate filaments, as delineated *in vitro*, is believed to progress through the parallel alignment of the rod domains of two monomers leading to dimers, which further associate in an antiparallel fashion to render tetramers, eight of which assemble into unit length filaments (ULFs) (Etienne-Manneville, 2018). Consecutive ULFs connect head to tail and compact radially to form the mature filaments (Etienne-Manneville, 2018).

In cells, GFAP hetero-oligomerizes with vimentin (Vim) to form the dynamic network that extends from the cell membrane to the nuclear periphery, while establishing multiple interactions with various cellular structures. Loss of function studies in mice showed that GFAP and vimentin in astrocytes partially compensate for each other (Eliasson et al., 1999), and thus, many revealing phenotypes of GFAP deficiency are apparent only in *Gfap*^{-/-} mice on a *Vim*^{-/-} background, i.e., in mice with astrocytes lacking both cytoplasmic intermediate filaments (reviewed in (Ridge et al., 2022)).

Both GFAP and vimentin are targets of a large variety of enzymatic and non-enzymatic posttranslational modifications that regulate their assembly and function (Etienne-Manneville, 2018). The GFAP and vimentin network establishes interactions with a multitude of proteins in various cellular structures and thus plays important roles in astrocyte migration, proliferation and organelle positioning, and homeostasis. Moreover, the GFAP-vimentin organization is key for the mechanical resistance of the central nervous system (CNS) to severe trauma, hypertrophy of astrocyte processes in CNS diseases, proper wound healing after brain and spinal cord trauma, determines neuroprotective functions of astrocytes in acute ischemic stroke, and affects post-stroke neuronal connectivity and functional recovery (reviewed in (Ridge et al., 2022)).

3. GFAP misfolding and aggregation in Alexander disease

In the intricate and vastly multifaceted context of the nervous system, the variety of GFAP isoforms, their homo- or hetero-oligomerization and different isoform ratios add additional complexity levels. Most of the work to date has been focused on the GFAP α isoform and its regulation, e.g. by phosphorylation or oxidative modifications (Inagaki et al., 1994; Viedma-Poyatos et al., 2020), while the same aspects remain either poorly analyzed or unknown for other GFAP isoforms. In this setting, the expression of ALXDRD mutant GFAPs further confound the picture. Importantly, the dose of mutant protein needed to perturb the wild type (wt) GFAP network poses a relevant issue, considering that even wt GFAP displays a propensity to misfold when expressed at high levels (Messing et al., 1998). The latest data suggest diverse expression ratios between wt and distinct mutant GFAPs to attain network disruption (Heaven et al., 2019; Kang et al., 2022; Yang et al., 2022), a subject that requires further investigation.

The appearance of cytoplasmic protein aggregates upon expression of ALXDRD mutants and their accumulation as ubiquitinated forms, already suggests a proteostasis problem, likely involving misfolding, misassembly and/or impaired degradation. While the similarity in sequence and secondary structure of vimentin and wt GFAP indicates an analogous assembly pathway from monomers into higher order oligomers and filaments, the monomer folding pathway has not been studied in detail for any GFAP isoform. Hence, a deeper knowledge of the GFAP folding pathway, its intermediates and their dependence on context factors, such as the redox state, metals, pH, etc., is required in order to fully comprehend the impact of the mutations. The folding pathway may be altered differentially by each mutation and, if this is the case, the intermediates formed may or may not allow progression into the final “correct” monomer structure. Unproductive intermediates, those that do not reach the final “correct” monomer structure, could just accumulate and associate with a variety of proteins, giving rise to aggregates that may not be necessarily identical in composition and pathogenic significance. Another putative scenario may arise when folding of mutant GFAPs progresses into the “correct” final monomer structure, but with local changes in charge, exposure or orientation of side chains that may disturb the subsequent assembly process.

4. Posttranslational modifications and protein interactions of GFAP and their alterations in Alexander disease

Detection of high molecular weight forms of GFAP ALXDRD mutants, resistant under various denaturing conditions, suggests the occurrence of various posttranslational modifications, potentially including crosslinks (Viedma-Poyatos et al., 2022; Yang et al., 2022), although their detailed characterization is still lacking. Phosphorylation of GFAP has been identified at several positions in the protein sequence and it should be considered that ALXDRD mutations may alter the accessibility of kinases and/or phosphatases to specific sites, in turn, changing the level and/or pattern of GFAP modification in the mutant forms. These alterations may affect another aspect of intermediate filaments, which is their dynamic nature and their subunit exchange that is regulated by phosphorylation. Fluorescence after photobleaching (FRAP) data have been obtained only for GFAP α and GFAP δ isoforms, the results indicating slower incorporation and dissociation of GFAP δ into the filament network (Moeton et al., 2016). However, to which point these aspects are altered by the presence of other GFAP isoforms or ALXDRD mutants remains largely unknown.

ALXDRD mutations may either eliminate or add target residues susceptible for posttranslational modification into GFAP, thus changing the posttranslational modification landscape and giving rise to proteoforms of unpredictable behavior. Mutants to cysteine, including R79C or R239C, causing severe forms of ALXDRD, imply a duplication of the cysteine content in GFAP, putatively conferring the network a higher susceptibility to disruption by oxidative posttranslational modifications (Viedma-Poyatos et al., 2022). In contrast, mutants to histidine may offer other possibilities as targets for new types of phosphorylation, lipoxidation or protein carbonyl formation. Additionally, crosstalk between diverse posttranslational modifications may be favored or precluded by the substitution of certain residues. In this line, the field lacks detailed information of the interplay between well-established posttranslational modifications identified in wt GFAP, such as phosphorylation, and non-enzymatic modifications, such as oxidation. Therefore, we are in a need of further proteomic studies to progress in the understanding of the consequences derived from ALXDRD mutations.

GFAP establishes a large variety of protein-protein interactions that are being collected in several databases (e.g. BioGRID, <https://thebiogrid.org/search.php?search=gfap&organism=all>). To date, the available information refers mainly to the GFAP α isoform, although some data concerning GFAP δ interactions are also available. Nevertheless, to the best of our knowledge, no interactions for other GFAP isoforms have been reported. Moreover, the consequences of these protein-protein interactions are generally poorly understood. In this line, ALXDRD associated mutants in any GFAP isoform may alter the wild type interaction network in ways that remain unknown. Hence, the need for interactomic studies in order to decipher the protein-protein interaction network for all the GFAP isoforms and their corresponding ALXDRD mutations, to achieve a real comprehension of the normal behavior and its alterations in the disease.

5. Impact of GFAP mutations on organelle function

Expression of certain ALXDRD mutants in cellular models also disrupts organelle homeostasis altering the positioning, morphology and function of cellular organelles, as well as astrocytic secretion (Jones et al., 2018; Viedma-Poyatos et al., 2022). Effects of ALXDRD mutants on organelles such as the mitochondria have been shown upon expression of some of these mutants in astrocytoma cells (Viedma-Poyatos et al., 2022). In this setting, oxidative stress was found, mainly due to increased generation of mitochondrial ROS (Viedma-Poyatos et al., 2022). Since mitochondria are key for ATP production, the oxidative stress induced by expression of the ALXDRD mutants may, in turn, affect the whole astrocyte metabolism (Fig. 1). Thus, imbalance between synthesis through the electron transport chain and glycolysis, whether total or partial, may not alter total ATP levels but lead to important consequences in other essential metabolic pathways, as well as in regulatory mechanisms. ATP is necessary for intermediate filament dynamics. Moreover, ATP is required to maintain the high glutathione levels found in astrocytes (8-10 mM), which are exported to the extracellular compartment where they are proposed to serve as the source of precursors for neuronal glutathione synthesis (reviewed in (McBean, 2017)). Although the precise mechanisms underlying this hypothetical glutathione “exchange” have not been elucidated, it can be envisioned that any alteration in astrocytic glutathione synthesis, would have consequences in the nearby neurons. Of note, expression of a GFAP R239C ALXDRD mutant construct in astrocytoma cells already causes a decrease in intracellular free thiol content (Viedma-Poyatos et al., 2022), mainly representing reduced glutathione (GSH) (Fig. 1). Whether this occurs in cells expressing other ALXDRD mutants, as well as *in vivo*, potentially affecting (nearby) neurons, deserves further research.

Changes in the redox potential due to oxidative stress can alter many aspects of cell function that rely on the introduction of posttranslational modifications in key proteins of signaling cascades (Corcoran and Cotter, 2013; Truong and Carroll, 2013), which subsequently may modify the final effector molecules (e.g. proteins, DNA) in diverse subcellular locations. In fact, these changes can alter both the levels and targets of posttranslational modifications in different organelles, leading to their anomalous function, location and/or morphology. This is achieved through the increased production of the modifying compounds (e.g. electrophiles such as 4-HNE) (Di Domenico et al., 2014; Martinez et al., 2008; Muntane et al., 2006) or by inducing the oxidation of the enzymes carrying out the modifications (e.g. kinases), in turn, leading to their

inhibition or activation (Behring et al., 2020; Byrne et al., 2020; Lim et al., 2020; Shrestha et al., 2020). In this context, changes in the astrocyte redox potential induced by the expression of certain ALXDRD mutants as described by Viedma-Poyatos et al. (Viedma-Poyatos et al., 2022) could impose a series of consequences derived from e.g. oxidative-regulated posttranslational modifications putatively including epigenetic changes that depend on intermediates of redox metabolism (Cyr and Domann, 2011), which need to be explored.

The protein misfolding and aggregation of ALXDRD mutants may be related to endoplasmic reticulum stress, a condition found in most neurodegenerative diseases, and impaired unfolded protein response (Alberdi et al., 2013; Clayton and Popko, 2016; Duran-Aniotz et al., 2017; Hoozemans et al., 2012; Smith et al., 2020)(Fig. 1). Additionally, altered vesicle trafficking leading to deficient ATP release has been described in cells expressing some ALXDRD GFAP mutants (Jones et al., 2018), in turn, affecting intercellular communications with neurons (by Ca^{2+} waves). Whether there is a direct relationship of the GFAP network and these mechanisms remains to be elucidated.

6. Factors contributing to adult onset of Alexander disease

Although mutant GFAP alone can promote a situation of increased stress, as deduced from the analysis of patient samples, cultured astrocytes and several models of ALXDRD (discussed in (Viedma-Poyatos et al., 2022)), additional sources of stress could contribute to the onset and/or progression of the disease. Several reports have related onset of adult ALXDRD or exacerbation of symptoms with endogenous or exogenous insults such as infections, fever, head trauma or excessive alcohol consumption (Messing, 2018b). These stressors have in common that they can result in oxidative damage to the affected tissue. In fact, oxidative effects of long-term alcohol consumption have been reported in the adult brain (Collins and Neafsey, 2012), whereas hyperthermia induces oxidative stress in the nervous system of animal models (Chauderlier et al., 2017). In addition, there is extensive literature about how oxidative stress derived damage occurring after traumatic brain injury affects the nervous tissue and can even hinder recovery (Rodriguez-Rodriguez et al., 2014). Therefore, oxidative stress could act as a potential inducer of the ALXDRD pathology in individuals carrying GFAP mutations that have been asymptomatic until adulthood. In this respect, it is noteworthy that astrocytes from *Gfap*^{-/-}*Vim*^{-/-} mice that lack cytoplasmic intermediate filaments, as well as astrocytoma cells expressing ALXDRD GFAP mutants, exhibit increased susceptibility to oxidative stress (de Pablo et al., 2013; Viedma-Poyatos et al., 2022)(Fig.1). Being a rare disease, establishing a causal link between these factors and ALXDRD would be challenging, and hence the experimental approaches to confirm such an association would be highly useful.

7. Experimental models of Alexander disease

Studies on ALXDRD have used astrocytoma cell lines and primary astrocytes available from different repositories to explore the impact of a variety of GFAP mutants in cell function (Yang et al., 2022), as well as their susceptibility to modification (Viedma-Poyatos et al., 2018; Viedma-Poyatos et al., 2022). In addition, knock-in mouse (Hagemann et al., 2006), fly (Wang et al., 2015), and zebrafish models (Candiani et al., 2020), as well as neurospheres (Gomez-Pinedo et al., 2017), induced pluripotent stem cells (iPSCs) and their isogenic controls (Battaglia et al., 2019; Gao et al., 2019; Jones et al., 2018; Li et al., 2018) started to become available for research in this field. In fact, these models allowed to establish the role of ALXDRD GFAP mutations in aspects such as altered glutamate transport (Tian et al., 2010), perturbation of organelle distribution (Jones et al., 2018), induction of autophagy (Tang et al., 2008a; Tang et al., 2008b) or intercellular communication with neurons (Wang et al., 2015). However, none of these models fully recapitulated all the aspects of the disease.

To tackle the aspects that remain obscure in the GFAP field and ALXDRD, the collection of experimental models already available is being expanded by the isolation of fibroblasts and derived iPSCs from additional patients carrying new GFAP mutations for further differentiation or reprogramming, the production of organoids, and animal models of ALXDRD. These models will likely contribute to a better understanding of the pathogenic mechanisms and to the design of protective or therapeutic strategies for this disease. For

detailed information on the experimental models available for the study of ALXDRD, see the recent review by Hagemann T.L. (Hagemann, 2022).

8. Therapeutic approaches

From the clinical perspective, in lack of treatment, the therapeutic approaches employed to date are symptomatic, and include antiepileptic drugs, supportive strategies and surgical procedures, such as shunt surgery in case treatment of a hydrocephalous is needed. Nevertheless, there is no standard treatment. As aberrant assembly is a common hallmark of mutant and excessive wt GFAP, most protective strategies explored to date have attempted to lower GFAP levels by targeting its expression. Among the compounds tested, phenytoin, carbamazepine and curcumin used in cellular models of ALXDRD normalized GFAP expression levels and improve folding and filament organization (Bachetti et al., 2012; Bachetti et al., 2021), while ceftriaxone decreased expression and induced elimination of mutant GFAP (Bachetti et al., 2010). Pexidartinib increased GFAP levels and decreased macrophage numbers in a mouse model of ALXDRD (Boyd et al., 2021). Other strategies for ALXDRD treatment were already discussed in several reviews (Hagemann, 2022; Messing et al., 2010). Since then, antisense oligonucleotides decreasing GFAP expression have shown beneficial effects in a recently developed rat model, which seems to be superior to mouse models (Hagemann et al., 2021), and has aided the design of the first clinical trial with ALXDRD patients (<https://clinicaltrials.gov/search/?term=Alexander%20Disease>).

8. Conclusions and future directions

In spite of the advances made to date and the availability of new experimental models for ALXDRD study, many aspects of the disease remain obscure or unknown. The role of the different GFAP isoforms and their impact on intermediate filament structure and function remain unknown. Furthermore, it is needed to decipher how ALXDRD mutations may impact the synthesis of the isoforms, their structure and their ability to incorporate into the network. The impact of oxidative posttranslational modifications on wt and the mutated GFAPs is only starting to be studied and, given the ever-increasing variety of cellular posttranslational modifications, this will require a large investment of time and energy to be fully deciphered. Effects on organelle positioning and function are also being slowly uncovered, but the number of ALXDRD mutations is large enough to require the full working force of several groups to get to a complete understanding of the subject. The development of an efficient therapy will benefit from the new data that are being obtained *in vitro* and from novel animal models.

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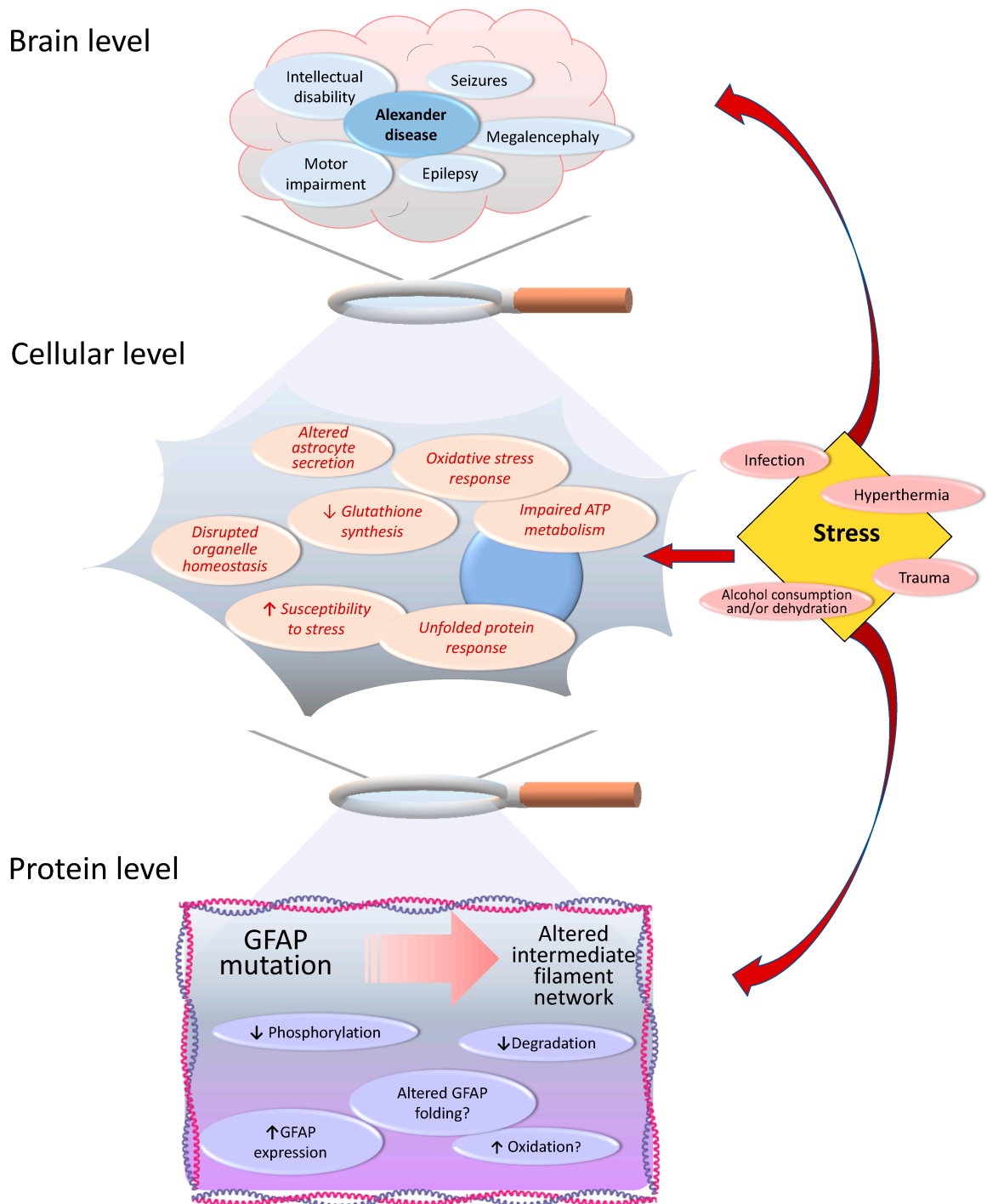


Figure 1. Main events occurring in AxD at the brain, cellular and protein levels, with special consideration of various types of stress as factors potentially involved in the pathogenesis or progression of the disease.

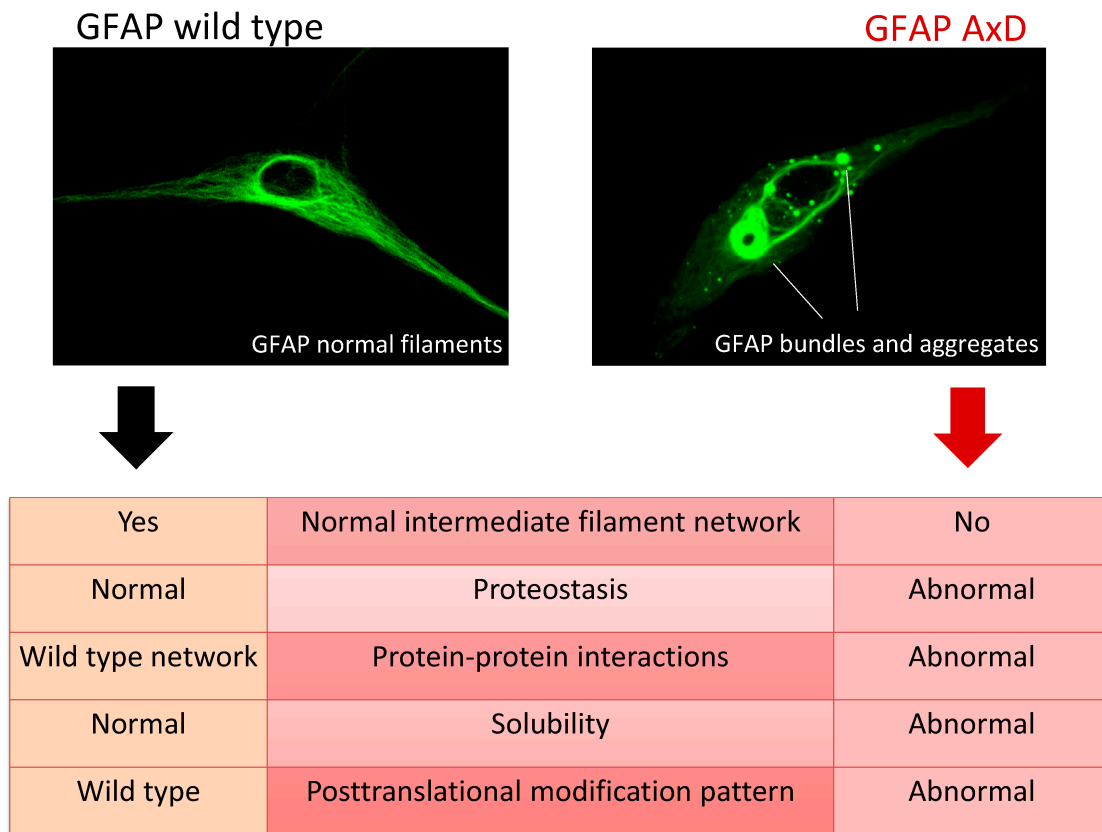


Figure 2. Comparison of the features of wild type and AxD mutant GFAP. Images at the top illustrate the morphological appearance of GFAP wild type filaments (left) and of the assemblies formed by a R239C AxD mutant (right), upon expression in a cellular model of AxD. Note the uniform appearance of the intermediate filament network (left) and the alterations, including bundles and aggregates, characteristic of cells expressing AxD GFAP, (right). The table at the bottom summarizes the main network and protein features under both conditions. For more details, please see (Viedma-Poyatos et al., 2022).

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