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Human Genome & Diseases: Review

Alexander disease: putative mechanisms of an astrocytic encephalopathy

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Abstract. Alexander disease (AXD) is the first primary astrocytic disorder. This encephalopathy is caused by dominant mutations in the glial fibrillary acidic protein (GFAP) gene, encoding the main intermediate filament of astrocyte. Pathologically, this neurodegenerative disease is characterised by dystrophic astrocytes containing intermediate filament aggregates associated with myelin abnormalities.
More than 20 *GFAP* mutations have been reported. Many of them cluster in highly conserved regions between several intermediate filaments. Contrary to other intermediate filament-related diseases, AXD seems to be the consequence of a toxic gain of function induced by aggregates. This is supported by the phenotype of mice overexpressing human GFAP. Nevertheless, GFAP null mice display myelin abnormalities and blood-brain barrier dysfunction that are present in AXD.
Given the pivotal role of astrocytes in brain physiology, there are many possibilities for astrocytes to dysfunction and to impair the functions of other cells. Physiopathological hypotheses are discussed in the frame of AXD.

Key words. Alexander disease; leukodystrophy; astrocytes; cytoskeleton; intermediate filaments; glial fibrillary acidic protein.

Introduction

Alexander disease (AXD) was first described as a clinico-pathological entity by Alexander in 1949 [1]. He described a 15-month-old boy affected with a rapidly progressing neurological illness associated with a hydrocephalus. The child's brain contained abnormal myelin, numerous 'fuschinophil bodies' and astrocytes displaying 'degenerative and proliferative changes'. It appeared clearly that 'these bodies are the result of fibrinoid degeneration in the fibres and cell bodies of the fibrillary neuroglia'. These bodies were identified as those previously described by Rosenthal in 1898 and termed Rosenthal fibres (RFs). Since then, several clinical forms of the disease have been delineated using pathological diagnoses, and ~100 cases have been reported worldwide. With modern neuroimaging techniques, the main diagnostic criterion is leukoencephalopathy with megalencephaly, but neuropathological evidence was still necessary to confirm the diagnosis until the discovery of mutations in the GFAP gene [2].
As all infantile and nearly all juvenile cases are sporadic, the underlying cause of AXD remained a mystery for a long time. However, rare familial cases [3–6] compatible with a dominant or recessive mode of inheritance suggested that AXD had a genetic origin. The subsequent finding of RFs in the brains of mice overexpressing the

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Table 1. Human diseases related to mutations in IFs and in IF-associated proteins.

	Proteins	Related diseases	Inheritance	References
IFs	GFAP	AXD	AD	[2]
	NF-L	axonal neuropathy (CMT 2E)	AD	[76]
	desmin	desmin-related myopathy/dilated cardiomyopathy	AD	[65]
	lamin A/C	Emery-Dreifuss muscular dystrophy	AD, AR	[161]
		limb-girdle muscular dystrophy type 1B	AD	
		dilated cardiomyopathy with conduction defect	AD	
		Dunnigan-type lipodystrophy	AD	
		axonal neuropathy (CMT 2B1)	AR	
		mandibuloacral dysplasia	AR	
	phakinin	cataract	AD	[162]
	keratin 1 and 10	non-epidermolytic and epidermolytic hyperkeratosis	AD	[69, 75]
	keratin 2A (2e)	ichthyosis bullosa of Siemens	AD	
	keratin 3 and 12	corneal dystrophy of Meesmann	AD	
	keratin 4 and 13	oral white sponge nevus	AD	
	keratin 6A and 6B	pachyonychia congenita	AD	
	keratin 17	pachyonychia congenita/steatocystoma multiplex	AD	
	keratin 5 and 14	EBS	AD, AR	
	keratin 9	epidermolytic palmoplantar hyperkeratosis	AD	
	keratin 16	non-epidermolytic palmoplantar hyperkeratosis	AD	
		pachyonychia congenita	AD	
	KRTBH 1 and 6	monilethrix	AD	
IFAPs	plectin	EBS with muscular dystrophy	AR	[66]
	αB-crystallin	desmin-related myopathy/cataract	AD	[65, 162]
	gigaxonin	giant axonal neuropathy	AR	[68]
	desmoplakin	dilated cardiomyopathy, woolly hair and keratoderma	AR	[67]

IFAPs, intermediate filament-associated proteins; NF-L, neurofilament light-chain; KRTBH, basic hair keratin; CMT, Charcot-Marie-Tooth disease; EBS, epidermolysis bullosa simplex; AD, autosomal dominant; AR, autosomal recessive.

human *GFAP* gene led to the discovery of dominant *GFAP* mutations in AXD. Hence, AXD became the first human disorder found to be related to an isolated and genetically defined dysfunction of astrocytes, and 1 of ~20 diseases caused by intermediate filament (IF) mutations (table 1). Over 50 patients carrying *GFAP* mutations have now been reported.

Several reviews have focused on the molecular aspects of AXD and the consequences of *GFAP* mutations on the astrocytic cytoskeleton [7–9]. Indeed, similarly to what is known about other IF-related diseases, *GFAP* mutations lead to the degeneration of astrocytes that specifically express this IF. However, the particularity of AXD is the secondary involvement of other cell types that do not express *GFAP*. This illustrates the importance of astrocyte functions in the central nervous system (CNS). The aim of our review is to describe the phenotype of AXD and to delineate physiopathological hypotheses regarding the effects of the accumulation of RFs in astrocytes and the consequences of *GFAP* mutations on other brain cells.

Description of AXD patients

Clinical presentation

Several clinical types of AXD have been delineated over the past 50 years on the basis of neuropathological data, i.e. widespread deposits of RFs throughout the brain and myelin abnormalities. The infantile type is the most frequent. The first signs appear between the age of 1 month and 2 years and comprise progressive megalencephaly, mental regression, progressive spastic paresis and epilepsy, which may be associated with ataxia and hydrocephalus [1, 10]. Acute episodes of intracranial hypertension are frequent. Death ensues within a decade. A neonatal form, characterised by its acuteness, hydrocephalus, seizures and death before 2 years, has been described [11]. In the juvenile type, cerebello-spastic degeneration and bulbar signs are frequent, but seizures and macrocephaly are not, whereas mental regression is variable [12–14]. The phenotype of pathologically diagnosed adult AXD is less clearly defined, ranging from a multiple sclerosis-like disease [15] to a tumor-like disease with neurodegeneration [6]. Other adults develop a degenerative disease that is similar to the juvenile type [4, 5], and similar to the phenotype of adult patients for whom the molecular diagnosis is provided [16, 17].

Molecular studies combined with magnetic resonance imaging (MRI) can be used to diagnose AXD in patients who do not display all of the phenotypic symptoms. Two AXD patients with macrocephaly and typical white matter abnormalities, but with no neurological signs have been reported [18]. The follow-up will probably confirm that the diagnosis was made at a presymptomatic stage of the disease. Mental retardation and white matter abnormalities with or without complex febrile seizures in nor-

mocephalic children can also be the main features of AXD, several years prior to neurodegeneration.

Radiological data

Since the invention of modern brain imaging, leukoencephalopathy with macrocephaly is the main criterion for the diagnosis of AXD. Tomodensitometry and MRI clearly reveal white matter abnormalities in children (fig. 1), but these may be absent or less prominent in older patients. MRI criteria were selected following the study of images from pathologically confirmed infantile/juvenile cases [19] and include (i) cerebral white matter abnormalities with frontal predominance; (ii) the presence of a periventricular rim of decreased signal intensity on T2-weighted images, thought to illustrate the presence of densely packed RFs under the ependymal epithelium; (iii) an abnormal signal of the basal ganglia and thalami; (iv) brain stem abnormalities; (v) contrast enhancement involving different brain areas (ventricular line or the underlying rim, frontal white matter, basal ganglia and thalami, brain stem), which is a marker of blood-brain barrier (BBB) disruption. Interestingly, the grey matter is not spared, as the basal ganglia and thalami exhibit an early hypersignal on T2-weighted images and progressive atrophy. Long-term brain-imaging studies revealed that white matter abnormalities progressively spread from periventricular regions to subcortical regions and from the frontal to the posterior lobes with time. The physiopathological process progresses in the course of the disease, resulting in a cavitation in the areas affected first. In juvenile/adult cases, radiological abnormalities are most obvious in the brain stem, cerebellum and upper cervical cord [14, 16, 17].

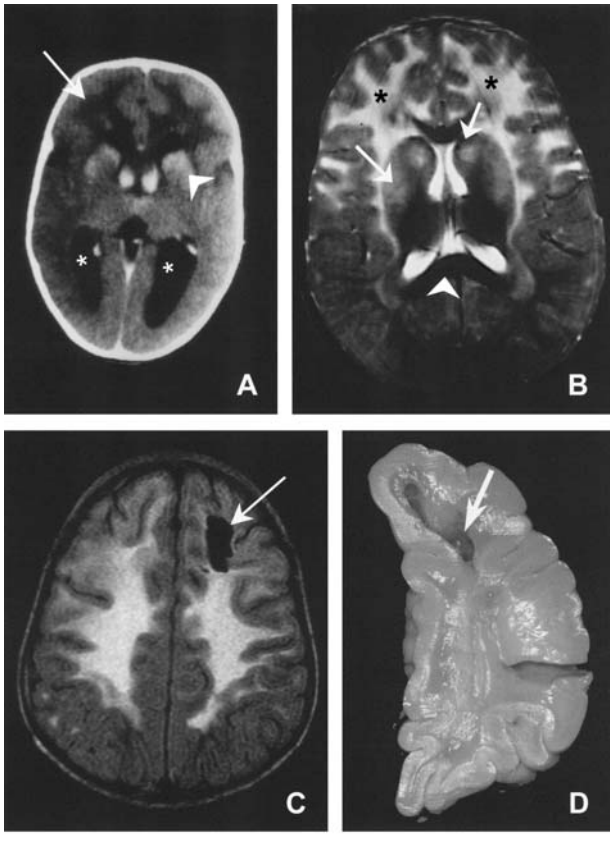

Figure 1. Radiological and macroscopic aspects of an infantile AXD brain. (*A*) Brain tomodensitometry with contrast enhancement. Enlarged lateral ventricles (occipital horns *). Hypodensity of the frontal white matter (arrow). Contrast enhancement of the basal ganglia (arrowhead). (*B*) Brain MRI (T2-weighted sequence). Anterior predominance of white matter hypersignal (*) and sparing of corpus callosum (arrowhead) and internal capsules. Hypersignal of basal ganglia (arrows). (*C*) Same patient as in *B*, brain MRI (FLAIR sequence). Presence of a frontal subcortical cavitation (arrow) surrounded by abnormal white matter in hypersignal. (*D*) Coronal section of a frontal lobe from an infantile AXD patient. Note the prominent cavitation (arrow).

Pathological data

The pathological signs used to diagnose AXD are the widespread presence of RF deposits within abnormal astrocytes and more or less pronounced myelin abnormalities. However, severity varies greatly, partly depending on the phenotype (infantile versus juvenile or adult).

The main macroscopic features are cavitating lesions of the subcortical frontal white matter and of the hilus of the dentate nuclei, enlarged lateral ventricles and stenosis of the aqueduct of Sylvius in the youngest patients [15, 20–24].

Gliosis of dystrophic astrocytes is the most characteristic microscopic finding [1] (fig. 2). Astrocytes are hypertrophied and express large amounts of IFs, GFAP and vimentin. They exhibit swollen processes and perikarya that contain RFs. RFs are insoluble rod-shaped hyaline and eosinophilic inclusions, the peripheries of which are weakly labelled by antibodies directed against GFAP, ubiquitin, αB-crystallin and heat shock protein 27 (HSP27), but not against vimentin [25]. Ultrastructurally, RFs appear as granular masses of osmiophilic material and are frequently connected with the astroglial cytoplasmic IF network [26]. They tend to accumulate in the endplates of astrocytes and form densely packed perpendicular arrangements around blood vessels, in the subependymal regions and in subpial zones. Astrocytes of the grey and white matters have the same dystrophic aspect, but the white matter is usually more intensely affected. These features are most marked in the brain stem and in the telencephalon, where they follow a rostro-caudal gradient of severity. Although RFs are not pathognomonic [27], the extension and the location of the deposits are highly suggestive of AXD.

The severe myelin changes found in most young patients together with relative axonal sparing make AXD a leukodystrophy. The severity of myelin abnormalities de-

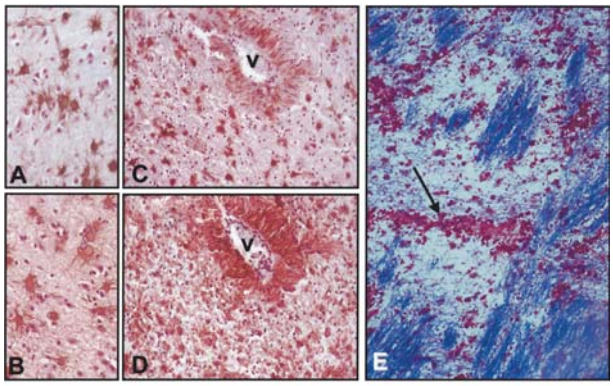

Figure 2. Pathological aspects of infantile AXD. (*A*, *B*, *C* and *D*) Paraffin-embedded sections of the brain of an infantile AXD patient (stained with anti-GFAP and anti-vimentin and revealed using the peroxidase/anti-peroxidase technique). (*A* and *B*) ×40. In cortical areas devoid of RFs, astrocytes are readily stained with both anti-vimentin (*A*) and anti-GFAP (*B*) antibodies. Note the abnormal aspect of the astrocytes (large cytoplasm, short and thick processes). (*C* and *D*) Adjacent slices, ×10. Conversely, RFs are numerous in the white matter. They are weakly labelled with anti-vimentin antibodies (*C*), but are clearly labelled with anti-GFAP antibodies (*D*). Note the crown of perpendicularly arranged RFs around the blood vessel (v). In sharp contrast to the cortex, the astrocytic somas are labelled with anti-vimentin antibodies (*C*), but rarely with anti-GFAP antibodies (*D*). (*E*) Cerebral white matter (×16). The paucity of myelinated fibers (blue lines) is evidenced by myelin staining (Luxol Fast Blue). RFs are disseminated (fuschia dots) and cluster around a blood vessel (arrow).

creases along a gradient from the anterior to the posterior pole of the telencephalon [3, 20, 21, 24]. In older patients, myelin loss may be patchy, limited to the periventricular white matter of the cerebrum [5] or restricted to the brain stem [14, 16]. These limited myelin changes challenge the belonging of adult AXD to the group of leukodystrophies. Contrary to astrocytes, oligodendrocytes appear normal. Markers of myelin destruction, such as myelin breakdown products and macrophagic infiltrates, have only been reported occasionally [12, 22]. Their scarcity may be indicative of dys- or hypomyelination rather than demyelination [3]. As radiological follow-up studies have shown the progressive nature of this leukodystrophy, the leukoclastic component of the disease probably progresses slowly, and myelin debris has probably already been eliminated by the time of autopsy. Some authors claim that there is a spatial relationship between the intensity of white matter abnormalities and RFs [3, 5, 10], whereas others have noted that demyelination can be limited even when RFs are homogeneously distributed throughout the CNS [20, 21].

Neuronal loss is often reported [5, 10, 20, 24], but axons are relatively well preserved in demyelinated areas [3, 26], except in cavitating lesions [10, 20].

In summary, it appears that the phenotype of AXD depends on the age of onset. The more precocious cases are generally severely affected, and the entire CNS rapidly degenerates, associated with intracranial hypertension, epilepsy, motor impairment, cognitive decline and extensive loss of white matter. Conversely, adult AXD progresses slowly, is characterised by predominant rhombencephalic degeneration, i.e. cranial nerve involvement, cerebellar and medullary atrophy, without epilepsy, cognitive impairment, and little, if any, white matter disease. Juvenile type AXD is an intermediate form.

Animal models

Spontaneous animal models of AXD

Encephalopathies resembling AXD have been reported in four dogs (reviewed in [28]) and one sheep [29] for which the GFAP gene has not been studied. In every case, the disease was characterised by rapidly progressing motor degeneration that appeared at or after the juvenile age, without cranial nerve palsy or epilepsy. This neurodegenerative disease also affected the littermates of two canine cases, suggesting a dominant mode of inheritance.

Mild ventricular dilatation was reported in two cases [28]. In all instances, numerous typical RFs were found in the CNS, particularly in the periventricular, subpial and perivascular areas. No astrogliosis was observed in the affected sheep [29]. Myelin loss was variable, but evident with typical histological staining. Surprisingly, the autopsy revealed a demyelinating neuropathy in one case.

The variability of the myelin abnormalities associated with diffuse RFs in these spontaneous animal models of AXD parallels that of human cases. As these animals were not maintained, genetically modified animals are the only experimental models available for the in vivo study of GFAP-related diseases.

GFAP knockout mice: deciphering GFAP functions

The study of several strains of GFAP knockout mice (GFAP–/–) [30–33] provided important data regarding GFAP functions (for recent reviews see [34, 35]). The most striking finding was that although GFAP is the main IF in mature astrocytes, it is not essential for global development. GFAP–/– mice have no particular clinical phenotype, live as long as wild-type mice and reproduce normally [30–32]. Moreover, the absence of GFAP does not modify the shape of astrocytes, and GFAP–/– astrocytes are capable of stellation when cocultured with neurons [34]. Importantly, GFAP seems to play a role in the astrocytic control of neuronal function and survival. These functions appear to be preserved in GFAP–/– astrocytes in vitro, as GFAP-deficient astrocytes cocultured with neurons still favor neurite outgrowth and neuron survival [36, 37]. However, in vivo, the lack of GFAP is associated with impaired hippocampal long-term potentiation [33] and cerebellar long-term depression [35].

The impact of the absence of GFAP is more evident in situations where the CNS of GFAP–/– mice is damaged [30, 38]. It has been suggested that GFAP provides protection against mechanical CNS injury [39], similarly to keratins in the epidermis. In ischemic conditions, GFAP seems to control the extension of brain damage [38, 40]. Consistent with this view, experimental allergic encephalitis is more severe in GFAP–/– mice than in wild-type mice, probably because GFAP forms a clearly defined edge to the inflammatory lesions in wild-type mice but not in GFAP-deficient animals [41]. In both situations, GFAP–/– astrocytes display a reactive gliosis that is not strictly identical to that of wild-type astrocytes. It is also noteworthy that ischemia and experimental allergic encephalitis are both accompanied by BBB disruption. Permeability studies with ^{125}I-labelled albumin revealed that the BBB in the spinal cord of old GFAP–/– mice was altered [32]. As the lack of GFAP in astrocytes prevents the induction of BBB properties in aortic endothelial cells [42], the pathological states affecting the BBB may have more repercussions in GFAP–/– mice because of impaired astrocyte-endothelial cell interactions. On the other hand, GFAP may have a protective role in stress conditions because it modifies the buffering capacity of astrocytes and their ability to metabolise extracellular glutamate. This is indirectly suggested by the finding of glutamine storage depending on the dose of GFAP in cultured GFAP–/– and GFAP+/– astrocytes [43], which is suggestive of impaired glutamine/glutamate metabolism. Importantly, GFAP–/– mice display late-appearing and asymptomatic myelin abnormalities [32]. Reduced myelination and altered oligodendrocyte morphology are visible in GFAP–/– mice after the age of 6–8 months, and oligodendrocytes resembling immature myelinating cells appear after 12 months. White matter atrophy with ventricular dilatation becomes obvious in 50% of animals after the age of 18 months.

In conclusion, the absence of GFAP seems to have more consequences in the challenged CNS, i.e. in pathological states or ageing, than in the resting CNS [34], suggesting that GFAP–/– mice would not have a normal life span in natural conditions. The results of studies on GFAP–/– astrocytes and animals do not immediately suggest a link between the lack of GFAP and AXD. Nonetheless, the occurrence of myelin abnormalities and limited disruption of the BBB in GFAP–/– mice is noteworthy in the context of AXD because it links GFAP function to the maintenance of myelin.

Mice overexpressing the human GFAP gene: the link between GFAP and the encephalopathy

In sharp contrast to GFAP–/– mice, mice overexpressing the human GFAP gene (hGFAP+) die from an encephalopathy at an age that is inversely correlated with the level of expression of the transgene [44]. Their brains contain many RFs, especially in perinuclear regions and processes of dystrophic astrocytes, but no myelin abnormalities have been reported. Furthermore, cultured astrocytes from hGFAP+ mice produce RFs [45].

The phenotype of hGFAP+ mice is obviously more similar to AXD than that of GFAP–/– mice. Importantly, it links the encephalopathy to the widespread deposits of RFs. The perturbations of the astrocytic cytoskeleton and the way in which astrocytes dysfunction in these mice and in AXD may be partly identical [46]. The absence of major myelin abnormalities in these animals is striking and reminiscent of adult AXD patients.

Molecular biology

Normal GFAP gene and protein

GFAP was first identified in the brain and is the main IF in astrocyte. In the developing brain, GFAP progressively replaces vimentin in the astrocytic IF network, although both IFs are coexpressed in reactive astrocytes and in some subpopulations of mature astrocytes, such as Bergmann glia [47]. GFAP is also present in glial cells of the peripheral nervous system and in several other organs, including liver, testis, kidney and bowel [48]. Despite the peripheral distribution of GFAP in these organs, RFs were not reported outside the CNS of AXD patients, but all these tissues were not systematically studied.

The human GFAP gene is located on chromosome 17q21 and contains nine exons spread over about 10 kb. Its main transcription product in astrocytes is a 2.7-kb messenger RNA (mRNA), called GFAPα, producing a 432-amino acid protein. Other mRNA isoforms were identified in human and in rodent that are expressed inside the CNS (astrocytes) and outside the CNS (nonmyelinating Schwann cells, hematopoietic tissues). Two minor isoforms expressed in the CNS lack exons in which mutations were reported, i.e. exon 1 for GFAPγ [49] and exon 8 for GFAPε [50]. It is not known whether the expression of these isoforms is modified in AXD patients' brains.

GFAP is a type III IF that shares a common tripartite structure with other IFs: a highly conserved α-helical rod domain flanked by non-α-helical and nonconserved N-terminal head domain and C-terminal tail domain. The rod domain is divided into four helical segments (coils 1A, 1B, 2A and 2B), separated by small linkers (linker 1, 12 and 2) (fig. 3 and 4). All of the α helices contain the repetitive heptad motif $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, with a predominance of hydrophobic residues at the buried first and fourth (*a* and *d*) positions and charged residues frequently at the fifth and seventh (*e* and *g*) positions [51]. Like other IFs, GFAP assembly starts by the parallel alignment of two monomers in register (fig. 3). The two-stranded coiled-coil structure is stabilized by the hydrophobic in-

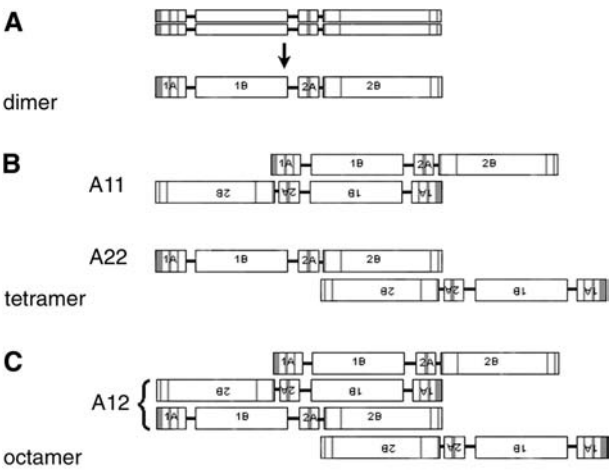

Figure 3. Putative model of GFAP assembly. Only the rod domains are drawn. Open boxes represent the four α-helical segments of the rod. Vertical bars in boxes illustrate the position of the mutations reported in AXD. Horizontal bars represent the small linkers. (*A*) Two monomers assemble parallel and in register to form a coiled-coil dimer. (*B*) Two dimers assemble antiparallel and partly staggered to form a tetramer following different modes of alignment. In the A_{11} mode, the 1B segments largely overlap, whereas the 2B segments largely overlap in the A_{22} mode. (*C*) Two tetramers assemble to form an octamer. According to the model proposed by Parry et al. [56], the 1B segment of a rod and the 2B segment of the opposite rod overlap following the third mode of alignment reported for tetramers (A_{12}). Note that in this model, most mutations cluster in regions that face segments of the opposite coiled-coil in which mutations were also reported. This suggests that these regions may be involved in the stabilisation of GFAP polymers.

terface between the α helices, and by ionic interactions [52]. Furthermore, distinct coiled-coil trigger sites within heptad repeat-containing amino acid sequences seem to be necessary to mediate coiled-coil formation [52]. The second step in IF assembly involves the association of a pair of dimers. Studies of paracrystals obtained with the rod of GFAP expressed in bacteria showed that two GFAP coiled-coils are aligned antiparallel and partly staggered following different modes of alignment, depending on the rod segments that overlap [53, 54] (fig. 3). Substantial data obtained with IFs other than GFAP suggest that tetramers associate laterally to form unit-length filaments that anneal longitudinally to produce loosely packed filaments [55, 56]. Then, extended filaments undergo an internal rearrangement, yielding mature and compact IFs [51]. IF polymers do not appear to be polarised; they can partially depolymerise and incorporate newly synthesised IF monomers on both ends, so that an equilibrium between IF subunits and polymerised IF is maintained [57]. Thus, IFs do not form a static network, but a dynamic and constantly remodelling network.

Different parts of IF monomers play different roles in polymer formation. The N-terminal 1A and C-terminal 2B regions of the rod are highly conserved among several human IFs (fig. 5) and among species, and play specific roles in dimer, tetramer and higher-order formations [51, 58]. The head and tail domains of GFAP contain motifs that facilitate filament assembly [54, 59, 60]. A con-

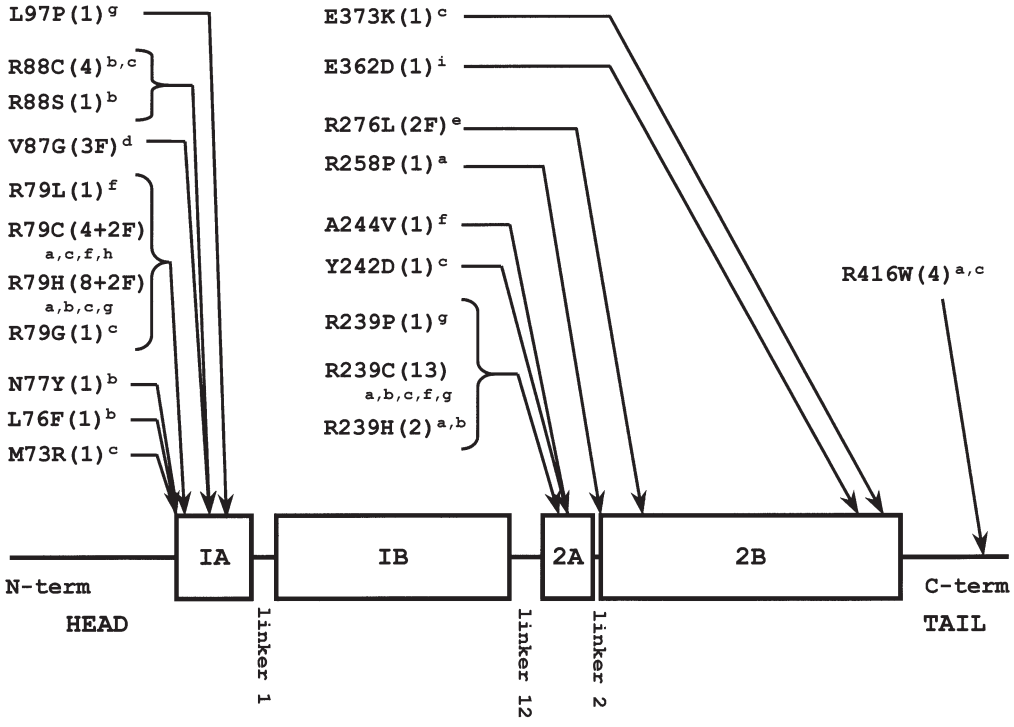

Figure 4. GFAP protein and mutations reported in AXD. The number of patients carrying each mutation is reported in brackets. For familial cases, the number of affected patients is noted, followed by 'F'. References: [2][a], [62][b], [18][c], [17][d], [16][e], [64][f], [63][g], [14][h], [13][i].

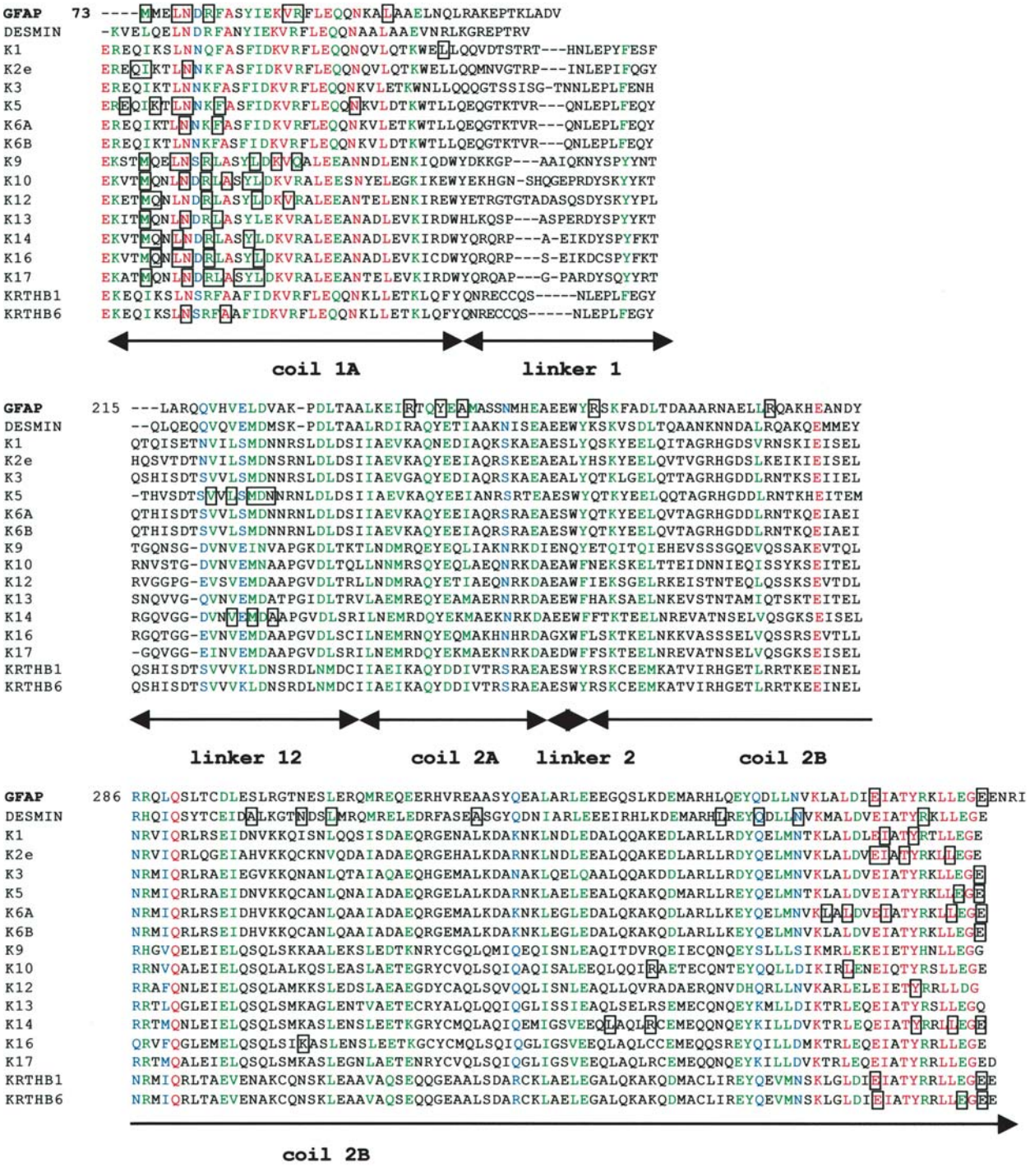

Figure 5. Alignment of the rod domain of different IFs (excluding coil 1B) showing the dominant missense mutations reported in IF-related diseases. IF sequences were obtained from the ExPASy server using the SWISS-PROT/TrEMBL database (http://www.expasy.org/), and sequences were aligned with CLUSTALW on the Pôle BioInformatique Lyonnais server (http://pbil.ibcp.fr/). K1 to K17, keratins 1–17, KRTHB, basic hair keratin. The amino acid numbering refers to GFAP only. Identical residues in all sequences in the alignement are in red font, conservative residues are in green, semiconservative residues are in blue. Boxed letters indicate amino acid substitutions reported in IF-related diseases.

served motif that could interact with molecular partners is also present in the tail domain [46].

GFAP gene mutations in AXD patients

As its product accumulates in RFs, the αB-crystallin gene was the first candidate for AXD, but no mutations were found in this gene in two patients with proven AXD [61]. Messing et al. produced transgenic mice overexpressing the human GFAP gene to determine the effect of GFAP upregulation in astrocytes. Some of these mice died at an early stage from a severe encephalopathy associated with RF formation [44]. Hence, the GFAP gene emerged as a candidate for AXD. DNA from patients with proven AXD was subsequently used to test this hypothesis, and mutations were found in most of them.

Since then, 57 patients with heterozygous dominant *GFAP* mutations have been reported (fig. 4). The GFAP gene was screened on the basis of neuropathological or radiological findings consistent with AXD, and mutations were found in 48 sporadic cases and 4 families [2, 13, 14, 18, 62–64]. In three patients in whom AXD was diagnosed on the basis of solid criteria, no mutations were found in the coding regions or in the intron-exon boundaries of the GFAP gene [2, 18, 62]. Parental DNA has been studied for most sporadic patients but no mutations were found. Therefore, sporadic AXD is caused by de novo mutations arising from germinal or post-zygotic events. This is in line with the finding of *GFAP* mutations in two pairs of monozygotic twins [63, 64]. Another sibship composed of two affected nontwin brothers was reported [16]. In this case, the parental DNA could not be tested, but both parents died in their seventies without neurological disease, suggesting that neither suffered from AXD. Thus AXD is likely caused by a germinal mutation in this family. Finally, inherited AXD was linked to the transmission of a mutant *GFAP* allele from a mother to her two sons [17]. In familial cases, the mutation segregates with the disease, confirming the dominant pattern of inheritance when the patients live long enough to reproduce.

Studies of 48 sporadic cases and 4 families revealed a total of 21 different amino acid substitutions, but no truncating or frameshift mutations have been reported. Two arginine residues, R79 and R239, account for 62% of amino acid substitutions in 52 pedigrees. These residues are both located in a region where mutations cluster: 50% of mutations are in the 1A segment of the rod domain comprising the R79 residue, whereas 35% of them are in the 2A segment comprising the R239 residue. As 73% of nucleotide substitutions occur in CpG dinucleotides where C is replaced by T or G is replaced by A, these mutations are likely the consequence of a deamination of 5-methylated C.

A phenotype-genotype correlation was attempted in the second series of AXD patients carrying *GFAP* mutations [62]. It was found that the R239C mutation resulted in more severe disease than R79H. This general view is supported by other genetic studies [14, 63, 64]. However, some mutations can cause both juvenile and infantile AXD [9]. Thus, more cases are needed to determine the link between genotype and phenotype.

AXD patients without *GFAP* mutations

Three AXD patients who do not carry a *GFAP* mutation have been identified. As overexpressed wild-type GFAP causes RFs in murine astrocytes, a dysregulation of GFAP production could result in an AXD phenotype. This could be either the consequence of a rearrangement of the genomic region containing the GFAP gene, or of a mutation in regulatory sequences. Alternatively, other genes may be implicated in AXD.

The NADH-ubiquinone oxidoreductase flavoprotein 1 (NDUFV1) gene, which encodes a subunit of the complex I of the mitochondrial respiratory chain, was screened in one patient without any GFAP gene mutations [18]. No mutations were found. This gene was screened because one patient exhibiting macrocephaly, encephalopathy, cystic degeneration of the white matter and myoclonic epilepsy carried a mutation in the NDUFV1 gene, suggesting that this gene could cause AXD. However, the diagnosis of AXD was not confirmed by radiological or pathological evidence in this case.

There is currently much interest in proteins that interact with the cytoskeleton in diseases involving IF aggregates with normal IF genes. Indeed, mutations have been identified in several genes encoding IF-associated proteins that mimic IF-related diseases: αB-crystallin in desmin-related myopathy [65], plectin in epidermolysis bullosa simplex with muscular dystrophy [66], desmoplakin in dilated cardiomyopathy with woolly hair and keratoderma [67], and gigaxonin in giant axonal neuropathy [68]. Thus, the few known IF-associated proteins that interact with GFAP are possible candidates for AXD patients without *GFAP* mutations.

Putative molecular consequences of *GFAP* mutations

The delineation of the consequences of *GFAP* mutations on the astrocytic cytoskeleton is just beginning. Many mutations are found in GFAP domains that are highly conserved among several IFs and play specific roles in the assembly of IF networks. With the notable exception of mutations reported in the desmin gene, another type III IF, the alignment of IF sequences related to human diseases shows that mutations in *GFAP* are homologous to many disease-causing *IF* mutations [9, 46] (fig. 5).

Many dominant mutations cluster in the N-terminal region of the coil 1A of GFAP and keratin genes. The

unique structural/functional role of coil 1A in promoting IF assembly probably explains the clustering of disease-causing mutations in this region [51]. The R79 in GFAP is homologous to an arginine residue in several keratins, the mutation of which causes different keratin-related human diseases (reviewed in [69]). Substitution of this arginine residue in keratin 14 by a non-basic amino acid leads to abnormal IF formation, possibly because it occupies a position that potentially stabilises the tetramer by forming ionic salt bonds between the two dimers [70]. A recent study on this mutant did not find an early assembly defect and suggested that either an abnormality in filament bundling occurs or that the interaction with cytoskeletal partners is inhibited [71].

Disease-causing mutations within the coil 2B domain have only been found in four AXD pedigrees, whereas clusters of mutations are found in this domain in most other IF-related diseases. Recent studies highlighted the importance of the conserved YRKLLEGEE motif in the C-terminal part of coil 2B in IF assembly [51, 72]. The *GFAP*-E373K mutation that involves one of the glutamic acid residues from this motif (YRKLLEGEE) has been detected in one AXD patient [18]. As this mutation modifies the charge of the residue, it may alter the properties of the glutamic acid residues clustering, thus affecting polymer formation. Conversely, the *GFAP*-E362D mutation [13] does not change the charge of the residue. Nevertheless, experimental data have shown that this highly conserved glutamic acid residue is essential for IF network assembly [72].

Finally, frequent mutations lying in the 2A segment seem to be unique to *GFAP*. It is possible that molecular partners specifically interact with this region of GFAP but not with the equivalent region of other IFs. The calcium-binding protein S100B binds to the N-terminal part of GFAP-coil 2A [73]. As S100B prevents GFAP assembly [74], mutations in coil 2A could impair GFAP-S100B interactions, resulting in the accumulation of GFAP polymers and possibly aggregates.

Molecular differences between AXD and other IF-related diseases

Like *GFAP* mutations in AXD, most disease-causing mutations found in IFs are dominant mutations, although rare cases with recessive inheritance have been reported for keratinopathies and laminopathies. Missense mutations in IFs that form obligate heterodimers (keratins) and homodimers (desmin) alter the formation and/or the maintenance of the IF network and lead to its collapse in the form of aggregates [65, 75, 76]. Both of these effects may be implicated in the physiopathology of the disease; the former would lead to a loss of function, and the latter would provide a basis for a possible gain of toxic function. A dominant loss of function probably underlies keratinopathies and desmin-related myopathy with desmin mutations, in which the cellular resistance towards mechanical stress is lost with the collapse of the IF network. This hypothesis is supported by the observation that knockout animal models and humans carrying homozygous truncating mutations precluding the expression of the IF [75] display a similar phenotype to classically affected patients. Conversely, the comparison of hGFAP+ and GFAP–/– mice phenotypes with AXD supports the toxic gain of function hypothesis owing to the presence of RFs [9].

A functional study of mutant GFAP (R79C, R239C and R416W) showed that they form dimers with wild-type GFAP with an affinity that is higher than wild-type GFAP itself [77]. This property implies a >50% chance of wild-type/mutant GFAP dimerisation, rendering probable the incorporation of mutant monomers within high-order GFAP polymers. Considering functional studies of desmin mutants [65, 78], GFAP mutants would be expected to exert a dominant-negative effect in astrocytes by inducing a collapse of the GFAP network. This was not verified by recent functional studies of R79C and R239H *GFAP* mutants transfected into human astrocytes. The overexpression of GFAP mutants causes the formation of abnormal coils of filament and inclusion bodies superimposed over an IF network composed of vimentin and GFAP 2 days post-transfection [79]. Although this study supports the toxic gain of function hypothesis, it does not imply that the remaining GFAP network is normal and functional and that it persists 2 days post-transfection. Thus, a loss of GFAP function could underlie some features of AXD in the background of a most evident gain of toxic function. This hypothetical loss of function could particularly account for myelin abnormalities and the disruption of the BBB observed in both AXD patients and in GFAP–/– mice, although these features are much more severe in AXD.

The loss of GFAP function could be masked or attenuated by the presence of vimentin. Indeed, pathological data (reference [9] and fig. 2) and functional experiments [79] suggest the persistence of a vimentin network in AXD astrocytes. Studies of GFAP–/– and vimentin–/– mice demonstrated that vimentin is necessary for the development of a normal GFAP network in astrocytes, although the precise nature of GFAP/vimentin interactions is not known [80]. Other experiments conducted with mutant GFAPs lacking parts of the tail or head domains further highlighted the GFAP/vimentin partnership. They clearly showed that the presence of vimentin can partially or completely restore a network containing mutant GFAPs that are incapable of forming a normal network on their own [60]. All these data suggest that vimentin could serve as a support for the maintenance of the remaining GFAP network in AXD astrocytes.

Answers to these questions will probably be brought in the near future to give insight into the molecular mecha-

nisms underlying AXD. If mutant GFAPs do not impede the first steps of IF formation, they may alter either the bundling of IF polymers or interactions with other molecular partners. As the normal GFAP network is not completely collapsed by mutant GFAP, it remains to be determined whether the remaining network is functional and, if not, whether the vimentin network reexpressed in AXD astrocytes can partially prevent and/or overcome the lost functions. Moreover, the noxious effects of GFAP aggregates in astrocytes have yet to be dissected.

AXD as an astrocyte-specific proteic inclusion body disease

Although RF deposits are diffuse in the brains of AXD patients, the gradual increase in their density between the caudal and the rostral poles of the cerebrum, which parallels the severity of myelin loss, is well documented in infantile cases. In some cases, RFs are present without myelin abnormalities, but myelin paucity without RFs has never been reported. The accumulation of RFs in dystrophic astrocytes is the most prominent pathological finding in hGFAP+ mice. These facts point to the potential pathogenicity of RFs and question the mechanism underlying RF-induced astrocytic degeneration.

Some authors have pointed out pathological similarities between AXD and proteic inclusion body diseases [8, 81]. This should probably be examined in more detail in the light of recent advances in our understanding of inclusion body diseases. In neuronal proteic inclusion body diseases, aggregates are frequently present within glial cells [82, 83]. Although the consequences of protein aggregates in astrocytes are not completely understood, they were suggested to precipitate the death of neurons that bear their own aggregates [84, 85]. In humans, the concomitant involvement of other cell types makes it impossible to measure primary dysfunction in astrocytes. Like neuronal inclusion bodies, RFs are ubiquitinylated and contain HSPs [25, 86]. They may behave in the same way as misfolded aggregated proteins in neurons. Protein aggregates are dually linked to the accumulation of oxidative stress markers in neuronal proteic inclusion body diseases: the production of misfolded proteins can lead to their oxidation [87], but the accumulation of aggregates is also facilitated by oxidative stress [88]. As oxidative stress markers accumulate in the brains of AXD patients [89, 90], this process may also occur in AXD. Although there are no experimental data supporting this hypothesis, the presence of RFs exceeding the capacity of the astrocytic protein folding machinery could be a primary event in the production of reactive oxygen species. Reciprocally, oxidative stress may favor the accumulation of RFs. The effects of RF accumulation in astrocytes are highlighted by the involvement of HSPs in the pathogenesis of proteic inclusion body diseases.

The expression of αB-crystallin and HSP27, which colocalises with RFs, is increased in the brains of AXD patients [25, 91], of hGFAP+ mice and in cultured RF-bearing astrocytes [45]. Their expression is also modified in several human proteic inclusion body diseases [92–94]. αB-crystallin and HSP27 are two small HSPs (sHSPs) [92, 95] belonging to the chaperone machinery and that bind and stabilise unstable conformers of other proteins [92, 96]. sHSPs have specific functions in the astrocytic IF network (reviewed in [97]). They interact with GFAP [55] and increase the soluble fraction of IFs [98], suggesting that they either modulate the assembly/disassembly cycle of GFAP or that they 'debundle' GFAP polymers [97]. Importantly, αB-crystallin can disaggregate RFs in vitro [99]. Therefore, αB-crystallin and HSP27 may be specifically produced in response to the accumulation of RFs in AXD. However, sHSPs have less specific anti-apoptotic properties and confer resistance to oxidant-induced cell death [100, 101], which may explain their beneficial activity in neuronal proteic inclusion body diseases [102]. Recent studies on neuronal proteic inclusion body diseases suggest that aggregates play a role in sequestering HSPs, leading to the depletion of anti-apoptotic and/or anti-oxidant activity, which in turn leads to neurodegeneration [93, 102]. Considering this scenario in the astrocytes of AXD patients, the accumulation of αB-crystallin and HSP27 in RFs could make them unavailable for anti-oxidant/anti-apoptotic purposes. This may damage the astrocytes themselves, as well as perturb the astrocytic functions, leading to encephalopathy.

Dystrophic astrocytes and the neighboring cells

Astrocytic reactivity is common in many brain diseases, but the specific role of astrocytes as primitive pathogenic cells in CNS damage has been demonstrated less frequently. *GFAP* mutations causing AXD induce myelin destruction, neuronal loss and BBB disruption. In this respect, AXD is the first disease to be described in which a dysfunction of astrocytes is the unique and primary cause of a CNS disorder. The pivotal role of astrocytes in organising and supporting the functions of other brain cells [103] opens a vast field of research in AXD physiopathology.

BBB

The BBB is a functional entity composed of a specialised microvascular endothelium, glial cell elements (astrocytic endfeet and macrophages) and a basement membrane. It provides an efficient barrier between the brain

parenchyma and the blood. This barrier maintains the homeostasis of the brain microenvironment and allows the active transfer of molecules or cells via paracellular and transcellular transporters [104, 105]. Experiments using endothelial cell/astrocyte cocultures and astroglial conditioned media showed that astrocytes can induce some BBB properties [105, 106]. Indeed, astrocytic endfeet form a lacework of lamellae closely apposed to the outer surface of the endothelium, giving anatomical support for exchanges between the two cell types [105]. Conversely, endothelial cells induce differentiation of astrocyte precursor cells [107] and can modify astrocyte morphology and pharmacology [108]. Thus, crosstalk between these two cell types is likely to underlie the induction and maintenance of the BBB, as well as adaptation to physiological and disease-related changes.

The BBB is disrupted in many CNS diseases, but its role in pathological processes has not yet been clearly defined, mainly because of methodological difficulty [106]. As mentioned above, there is considerable evidence suggesting that astrocyte-endothelial cell interactions are impaired in GFAP knockout mice. The disruption of the BBB in AXD is revealed by the MRI contrast-enhancement of several brain areas. The extraordinary accumulation of RFs in astrocytic endfeet on the parenchymal edge of cerebral blood vessels is an obvious pathological substrate for impairment of the BBB, although the only ultrastructural alteration reported so far is a reduced number of pinocytic vesicles [23]. These data suggest that impairment of the GFAP network in AXD astrocytes alters BBB functions, which does not rule out the possibility that astrocytic RFs have an indirect toxic effect on the BBB.

Glutamate uptake, energy supply and free-radical scavenging

Astrocyte metabolism is tightly linked to the metabolism of other CNS cells, particularly in terms of energetic substrate production, glutamate metabolism and anti-oxidant activity. Many studies have looked at the relationships between astrocyte and neuron metabolism, whereas fewer have looked at the relationships between astrocyte and oligodendrocyte metabolism. An alteration of this key astrocytic function could cause both myelin and neuronal damage in AXD.

Mounting evidence suggests that astrocytes produce and deliver energetic substrates and neurotransmitter precursors to neurons and take up potentially deleterious glutamate and ammonia from extracellular spaces [109, 110]. Therefore, the pathways used to transport neurotransmitters and metabolites between astrocytes and neurons are major pathways whereby astrocyte metabolic activity is linked to neuronal synaptic activity. Relatively little is known about astrocyte-oligodendrocyte metabolite exchanges. Oligodendrocytes probably use astrocyte-derived lactate as an energy source [111] and possess AMPA/kainate receptors implicated in the differentiation of precursor cells [112, 113]. Neurotransmitter metabolism is probably one of the main functions of astrocytes related to CNS diseases. Alteration of astrocytic glutamate metabolism is involved in CNS diseases comprising a prominent inflammatory component (experimental allergic encephalitis [114], human T-lymphocytotropic virus type-1 infected astrocytes [115], multiple sclerosis [116]), in hyperammoniemic states [117], in neuronal proteic inclusion body diseases (Huntington's disease [118], SOD1-linked amyotrophic lateral sclerosis [119]) and in hypoxic/ischemic conditions [120, 121]. This process results in excitotoxic injury of vulnerable cells expressing AMPA/kainate-type glutamate receptors, i.e. neurons and oligodendrocytes [122–124]. Excitotoxicity has not been studied in AXD, but this would be interesting given the growing importance of this mechanism in CNS diseases involving neuron, oligodendrocyte and myelin damage. As astrocytic glutamate transporters are oxidant vulnerable [125, 126], excitotoxicity is linked to oxidative cellular injury [127], which is another aspect of interest in the astrocytic metabolism of AXD brains.

Antioxidant activity defects are another potential astrocyte-dependent mechanism of cellular injury in the CNS. This could be the case in AXD patients in whom markers of oxidative stress are present (see above). Astrocytes display a high anti-oxidant capacity, suggesting that they might supply anti-oxidant molecules to other cells [128, 129]. The vulnerability of CNS cells towards oxidative stress means that perturbation of the astrocytic anti-oxidant function probably has deleterious consequences on the function and survival of other cells. The oxidant susceptibility of oligodendrocytes has been demonstrated [130–132] and is thought to be related to their relatively low anti-oxidant defence and to the oxidant-vulnerable lipid content of myelin [132, 133]. Interestingly, astrocytes protect against oxidative stress-induced oligodendroglial death in cocultures [134, 135]. This raises the hypothesis that astrocytes fail to prevent oxidative white matter and/or neuronal damage in AXD, which, as discussed above, can be combined with the excitotoxic hypothesis.

Astrocyte network-neuronal network interactions

Epilepsy is a frequent feature of AXD. Besides the data discussed above, what is the basis for impaired neuronal activity in this astrocytic disease? It has been demonstrated that complex crosstalk occurs between two parallel networks within the CNS. The first network is composed of neurons communicating via synaptic transmission; the second comprises astrocytes that interact via gap junctions formed between adjacent cells with connexin

43, as well as by extracellular signals (prostaglandins, ATP) [136, 137]. Neurotransmission influences the functional astrocytic syncytium by eliciting local increases in the intracellular calcium concentration that are susceptible to spread in the form of calcium waves in the glial network. Neurons also modulate the number of astrocytic gap junctions [138], hence controlling the neurotransmission-induced astrocytic activation. In response to neuronal activity, an increase in cytoplasmic calcium in astrocytes triggers the release of glutamate, D-serine and nitric oxide, which in turn affects neuronal synaptic activity. Finally, astrocytes control the number of functional synapses [139] and can release S100B, which modulates long-term neuronal synaptic plasticity [140].

We do not yet know the extent to which impairment of the reciprocal control of astrocytic and neuronal networks underlies CNS diseases. It is worth mentioning that mutations in the human connexin 43 gene underlie oculodentodigital dysplasia [141], the symptoms of which may include epilepsy [142]. This suggests that impairment of the astrocytic network can have consequences on synaptic transmission (the white matter is not spared in this disorder, see below). Astrocytic connectivity remains to be tested in AXD and in astrocytes expressing mutated GFAP to investigate possible consequences on the neuronal network. There is some evidence implying that astrocyte-astrocyte and astrocyte-neuron communication is disrupted in AXD. First, oxidative stress markers are present in the brains of AXD patients, which could block glial coupling [143]. Second, RFs accumulate in glial processes, and GFAP is expressed in close proximity to synapses. Third, synaptic activity is altered in GFAP–/– mice (see above), and GFAP phosphorylation is controlled by glutamate [144], suggesting that GFAP plays a role in the astrocytic control of synapses. The astrocytic S100B protein, which promotes GFAP disassembly [74, 145], is a possible link between the astrocytic GFAP network and synaptic activity. As the modification of other components of the astrocytic cytoskeleton is related to neuronal synaptic activity [146], the modulations of IF disassembly may also be implicated in the astrocytic control of synapses, and GFAP aggregates may impair such mechanisms.

Specific astrocyte-oligodendrocyte interactions

Myelin involvement in AXD highlights the importance of astrocyte-oligodendrocyte interactions in the formation and maintenance of myelin. In this respect, AXD is probably the first human disease to be described in which astrocyte dysfunction is the only factor precipitating myelin destruction.

The main function of oligodendrocytes is the formation of myelin sheaths around axons of the CNS. The sheaths are interrupted at the nodes of Ranvier, which allow the saltatory conduction of action potentials. The unique composition of myelin ensures the electrical insulation of axons, whereas its segmental structure accelerates the conduction of nerve impulses, thereby ensuring that the signal is transferred correctly over long distances and saving space and energy [147].

Astrocyte-oligodendrocyte interactions are of utmost importance in oligodendrocyte differentiation and survival, and in each step of myelin sheath formation, maintenance and repair [103, 147]. In vitro experiments showed that astrocytes favor the migration of oligodendrocyte progenitors via adhesion molecules [148] and are required for the expression of maturation markers by oligodendrocyte lineages [149, 150]. Myelinogenesis is facilitated in vitro by astrocytes [151], which also promote the outgrowth of oligodendrocyte processes [152] and the interaction between oligodendrocyte processes and axons, a crucial step in the initiation of myelination [153]. The experimental induction of demyelinating lesions in vivo proved the importance of astrocytic factors in remyelination [154–156]. Astrocytes and oligodendrocytes communicate via gap junction-mediated contacts (connexin 43) [157], and several soluble astrocyte-derived signals promoting oligodendrocyte survival have been identified (platelet-derived growth factor, insulin-like growth factor 1, ciliary neurotrophic factor and leukaemia inhibitory-like protein [135, 158, 159]).

The mechanisms underlying the failure of astrocytes to promote myelin formation, myelin maintenance and myelin repair are poorly known. White matter abnormalities have been reported in oculodentodigital dysplasia [142], in which an impaired crosstalk between the two cell types is plausible. The importance of the communication between astrocytes and oligodendrocytes was recently demonstrated in multiple sclerosis, in which the abnormal expression of Jagged 1 by reactive astrocytes is responsible for the failure of myelin repair following myelin destruction [160].

Myelin abnormalities in AXD and in GFAP–/– mice illustrate the role of GFAP in myelin maintenance. Further studies should provide insight into the importance of astrocyte-derived signals in AXD and in other diseases affecting the white matter.

Conclusion

Although AXD was identified more than 50 years ago, its phenotype is still being delineated thanks to the genetic analysis of patients with atypical presentations. It is now clear that AXD is a primary astrocytic disease and that its manifestations are the result of astrocyte dysfunctions leading to both myelin damage and neuron dysfunction.

The discovery of the morbid gene is the first step in understanding the physiopathology of AXD. The accumula-

tion of GFAP in the form of insoluble RFs seems to be the starting point of the degenerative process. Basic issues such as the effects of GFAP mutants in astrocytes, the behavior of RF-bearing astrocytes in vivo, and the consequences of an altered GFAP network and of the presence of potentially toxic RFs remain to be clarified.

The consequences of astrocytic dysfunction on brain homeostasis are partly known and are highlighted by Alexander disease, which is the only IF-related disease in which nearby cells that do not bear aggregates are clearly damaged. The diversity of astrocyte functions means that there are many hypotheses about AXD physiopathology. These may help us to understand other brain diseases, particularly leukodystrophies. Future studies will probably focus on several of these aspects, with the aim of identifying the crucial steps in the degenerative process and developing therapeutic strategies for the treatment of this devastating neurological disease.

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