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# Neuroglial patterns are shared by cerebella from prion and prion-like disorder affected patients

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ABSTRACT

Neurodegenerative diseases, such as Alzheimer's and Parkinson's, are considered prion-like disorders because they are all proteinopathies in which aberrant proteins spread throughout the brain during disease progression. The overall aim of this study is to determine how glial cells are commonly involved in the neurodegeneration progress observed in all these pathologies. The suggestion that they are cell types in which prion and prion-like disorders have common behaviour is the hypothesis on which this study is based. Morphological and distribution differences in astroglial and microglial cells in the cerebellum from prion and prion-like disease-affected patients were assessed here by histopathological and immunochemical tools. To our knowledge, this is the first study to focus on the comparative assessment of glial profiles in these human brains. Activated microglial population was demonstrated in both, prion and prion-like disorders, although in higher extent in the first. In astroglial activation, specific patterns of alterations suggesting both degenerative and potentially neuroprotective or restorative stem cell response, were shown to be alternatively shared by cerebella from all disorders studied. Neuro-protective strategies for these disabling disorders are particularly desirable.

## 1. Introduction

Currently, the association between neurodegenerative processes and neuroinflammation is undeniable. The presence of reactive microglia, release of cytokines, alteration of the blood brain barrier (BBB) and even neuronal death are hallmark elements belonging to the neuroinflammation hypothesis (Estes and McAllister, 2014). This neuroinflammation always appears to be associated with neurodegenerative diseases without the presence of external immune cell infiltration (Estes and McAllister, 2014; Filiou et al., 2014; Crotti and Glass, 2015). All of these features are directly or indirectly related to glia, giving this cell population a key role in the neurodegenerative process (Pasqualetti et al., 2015; Ransohoff, 2016). The glial population includes several heterogeneous cell populations. Based on their links to the immune response, studies regarding this hypothesis have mainly focused on microglia and astroglia (Verkhratsky et al., 2016; Liddelow et al., 2017a; Carroll and Chesebro, 2019).

As the main immune cell of the central nervous system, microglia are considered a critical player in neuroinflammation, and their activated phenotype is a specific marker of neurodegeneration. On the other hand, astroglial cells support the cells that form the BBB, establish close contact with neurons and play a regulatory role with microglia (Boche et al., 2013a; Pekny et al., 2016; Reichenbach and Bringmann, 2017; Verkhratsky and Nedergaard, 2018). Their activation is always observed in neurodegenerative tissues, and recent works have associated it with neurotoxicity (Ransohoff, 2016). However, the role of astrocytes in neuroinflammation is often understated (Jiang and Cadenas, 2014).

Currently, some authors suggest that several neurodegenerative disorders, such as Alzheimer´s, Parkinson´s and Huntington's disease, frontotemporal dementia and amyotrophic lateral sclerosis, should be grouped together. They present molecular mechanisms of specific protein aggregation and spreading similar to those demonstrated for aberrant prion proteins. As this is typical of the pathological proteins found in prion diseases, they are all included in the same cluster called prion-like disorders (Prusiner, 2013). All of them have fatal outcomes. Palliative or medical treatments have been evaluated during recent decades without conclusive results. Therefore, there is an evident lack of knowledge of the pathogenesis of these brain disorders (Prusiner, 2012; Fernandez-Borges et al., 2013; DiSabato et al., 2016).

*Abbreviations:* BBB, blood brain barrier; CJD, Creutzfeldt-Jakob disease; AD, Alzheimer´s disease; MND, motor neuron disease; PD, Parkinson´s disease; HD, Huntington´s disease; FTD, frontotemporal dementia; HE, haematoxylin - eosin staining; GFAP, glial fibrillary acidic protein; CD68, cluster of differentiation 68; MHCII, major histocompatibility complex class II

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As gliosis is broadly known as a chronic insult found in the brains of patients affected by proteinopathies, by focusing on glial populations, neurological progress may be more fully understood. The purpose would be to finally find a therapeutic target.

Efforts that have begun with this aim using natural Scrapie as a model of prion disorders have provided very interesting findings. A close relationship between astrogliosis and the neuropathological features associated with prion diseases was evident. An indisputable involvement of astrocytes in prion progression was also demonstrated (Sarasa et al., 2012; Hernandez et al., 2014). Based on these outstanding findings, the present study presents a novel morphological approach that suggests glia as a cell type with similar behaviour in prion and prion-like disorders.

The specific objective of this study is to compare the astroglial and reactive microglial profiles present in cerebellar sections from patients affected by prion and prion-like diseases to more fully understand the potential role of glia in the neurodegenerative process characteristic of all these pathologies. To determine whether specific glial patterns are in common found in all neurodegenerative diseases is intended here.

The cerebellum is not the primary damaged region in most of these disorders because each one has been mainly associated with other brain regions. However, it has been selected on the basis of consideration of that pathologies examined here affect the whole central nervous system (Sjobeck and Englund, 2001; Sebeo et al., 2004; Mirdamadi, 2016). Moreover, this region, although resistant to progress of some neurodegenerative diseases, is considered to be prone to injuries based on anatomical and physiological basis in some others (Sarna and Hawkes, 2003). It also seems to be a reference point to detect neuroinflammation (Lyoo et al., 2015).

Despite glial alterations are known to be associated with several neurodegenerative diseases, there is little information about the cerebellum. Furthermore, although evidences about heterogeneity and diversity of status of activated microglia and astrocytes during neurodegeneration have been reported, to our knowledge, studies focused on similarities of glial variations shared by all these diseases have been not performed.

## 2. Material and methods

### 2.1. Materials

The present study was performed on samples provided by several banks from the UK brain tissue network following the national legislation guidelines on this matter. The samples came from the Centre for Clinical Brain Sciences, University of Edinburgh (cases affected by Creutzfeldt-Jakob disease, CJD, Alzheimer´s disease, AD and motor neuron disease, MND), Centre for Neuroscience, Parkinson's UK Brain Bank (cases affected by Parkinson´s disease, PD) and the Institute of Psychiatry, London Neurodegenerative Diseases Brain Bank (cases affected by Huntington´s disease, HD and frontotemporal dementia, FTD). Additional samples were used as non-dementia controls (Table 1).

A total of 21 fixed sections corresponding to cerebellar sagittal samples at the vermal level, from molecular to white matter layers, were studied. They corresponded to three different affected cases from each of the pathologies at the final stage.

The studies developed for these samples were approved by the Ethical Committee for Clinical Research of the Government of Aragón (CEICA; REFERENCE NUMBER: PI 15/0036, Acta Nº 05/2015).

### 2.2. Methodology

Three - five millimetre slices from each individual were paraffin-embedded, from which 5 µm were sectioned and histologically (haematoxylin - eosin staining, HE) or histochemically processed after formic acid treatment for prion inactivation (1 h) on all occasions.

**Table 1**
Data provided by respective Brain Bank corresponding to patients affected by each disease.

| clinical case | AGE | SEX | NEUROPATHOLOGICAL DIAGNOSIS |
|---|---|---|---|
| CONTROL 1 | 69 | Female | |
| CONTROL 2 | 63 | Male | |
| CONTROL 3 | 74 | Male | |
| FTLD 1 | 78 | Female | TDP-43 subtype C |
| FTLD 2 | 69 | Male | TDP-43 subtype C |
| FTLD 3 | 74 | Male | TDP-43 subtype C |
| HD 1 | 63 | Male | Vonsattel grade III |
| HD 2 | 48 | Female | |
| HD 3 | 70 | Male | Vonsattel grade III |
| sCJD 1 | 70 | Male | MM2 |
| sCJD 2 | 78 | Female | MM1 |
| sCJD 3 | 74 | Male | VV2 |
| PD 1 | 79 | Male | Idiopathic PD and possible Lewys dementia |
| PD 2 | 89 | Female | PD with a synucleinopathy and low AD |
| PD 3 | 84 | Male | PD with a synucleinopathy |
| AD 1 | NA | NA | Braak 3+ Lewys bodies Dementia |
| AD 2 | NA | NA | Braak 4 |
| AD 3 | NA | NA | Braak 6 |
| MND 1 | NA | NA | MND |
| MND 2 | NA | NA | MND with dementia |
| MND 3 | NA | NA | MND + FDTL |

FTDL: Frontotemporal Dementia; HD: Huntington´s Disease; sCJD: sporadic Creutzfeldt-Jakob Disease; PD: Parkinson´s Disease; AD: Alzheimer´s disease; MND: Motor Neuron Disease.
NA: Not available.

After HE staining, all samples were assessed by light microscopy to identify neuropathological lesions.

#### 2.2.1. Misfolded protein staining

Specific protein immunostaining was performed on all samples to verify the diagnosis of each case (Fig. 1). To detect aberrant proteins immunohistochemical (IHC) protocols were applied following Manufacturer's instructions for each antibody used. It was necessary to include an epitope unmasking protocol, consisting of 96% formic acid immersion (5 min) and steam bath in distilled water (10 min). Incubation was performed with the corresponding primary antibody that recognizes β-amyloid (6 F/3D at 1/500 dilution; Dako, Glostrup, Denmark), α-synuclein (NCL-L-ASYN at 1/500; Leica Biosistems, Newcastle, United Kingdom), huntingtin (mEM48 at 1/100; Merck Millipore, Darmstadt, Germany) or TDP-43 (TDP-43 Rabbit Policlonal antibody at 1/500; Proteintech, USA) for 1 h RT, except for mEM48 that required overnight incubation. Then, EnVision™ mouse polymer (DAKO, Glostrup, Denmark) was used as a visualization system for 30 min RT in all cases.

For PrPsc detection, protocol previously described by Monzón et al. (2018) was applied.

#### 2.2.2. GFAP immunolabelling

After endogenous peroxidase inactivation by incubation of these sections with peroxidase blocking reagent (5 min; DAKO, Hamburg, Germany), the sections were incubated with a primary antibody against glial fibrillary acidic protein (GFAP, 1/500 for 30 min RT; DAKO), followed by an incubation with enzyme-conjugated polymer Envision™(30 min, RT; DAKO). Then, DAB PLUS (10 min; DAKO) was used as the chromogen. Sections were counter-stained with haematoxylin (DAKO).

#### 2.2.3. Reactive microglia detection

The protocol used for reactive microglia labelling was the same as that applied for GFAP, with the exception of the primary antibody. In this case, CD68 (1/500, 30 min RT; DAKO) and MHCII (1/200, 30 min RT; DAKO) were used.

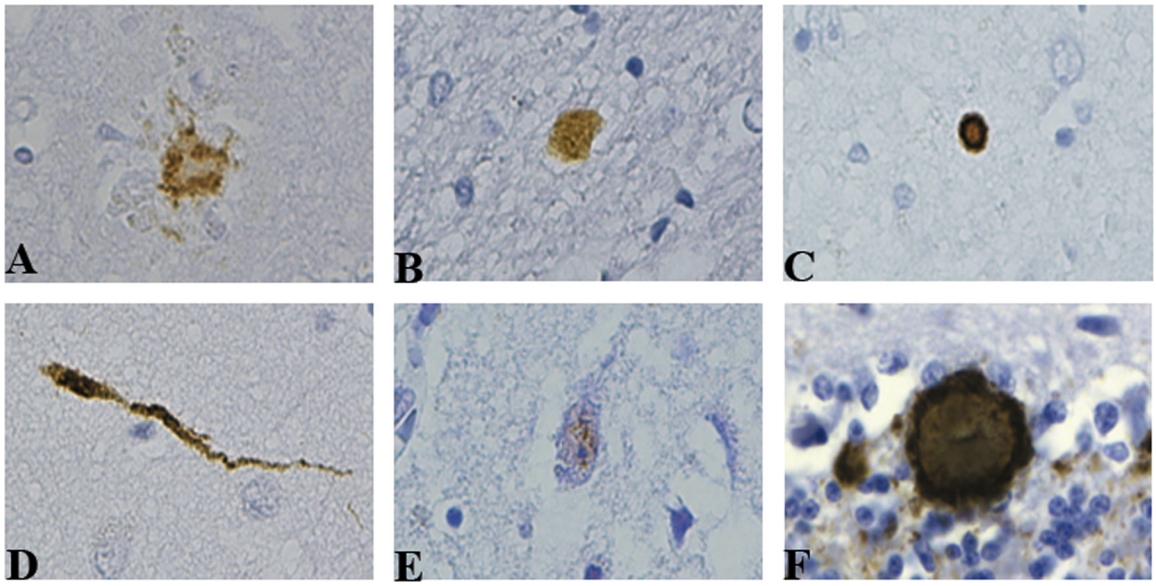


**Fig. 1.** Misfolded protein immunostaining detected in samples from an (A) Alzheimer´s disease, AD, (B) Parkinson's disease, PD, (C) Huntington´s disease, HD, (D) Frontotemporal dementia, FTD, (E) Motor neuron disease, MND and (F) Creutzfeldt-Jakob disease, CJD - affected patient by using the corresponding antibody against β-amyloid, -α-synuclein, huntingtin, TDP-43 and prion protein, respectively.

*2.2.4. Light microscopy examination*

Both GFAP- and reactive microglia-immunolabelled sections were thoroughly assessed in each cerebellar layer for morphology and distribution variations in the labelling. After immunohistochemical processing, all marker immunostaining was scored (following previously described semi-quantitative method) from negative (−) to maximum (+ + + +) by evaluating the density and the extension of the labelling deposits by light microscopy in each layer from each cerebellum.

*2.3. Statistical analysis*

Rating data were entered into an Excel spreadsheet and analysed using Statistical Package for Social Sciences (SPSS) software (version 22.0).

The Kruskal-Wallis test was performed for analysis. As significant differences between the groups were detected, *post hoc* testing was performed to identify the groups with respect to controls which significant differences existed (Scheffe).

## 3. Results

The main findings observed by histopathological examination are described below.

Degeneration of the granular layer by decreased number of granule cells was found in three of the cases studied (from MND, FTD and control patients, respectively; Fig. 2A).

Moderate or severe damage of Purkinje cells and hypertrophy (with or without swelling) of their main dendritic tree were very often observed (Fig. 2B).

Spongiosis always appeared in the CJD cerebella (Fig. 2C). Moreover, this histopathological lesion could also be observed in some prion-like samples (although presenting a more irregular morphology, distinguishable from that typically present in CJD cases; Fig. 2D). Meanwhile, the control samples did not show vacuolation (Fig. 2E).

Most samples showed protein aggregates mainly in the molecular and Purkinje layers but also in granular layers (the latter at a lower frequency).

An outstanding and constant finding consisted of astroglial immunostaining detected in intimate association with protein deposits in all occasions (Fig. 3).

Although significant differences were found only for the HD group ($p < 0.05$; although $p = 0.07$ for CJD group), an increase in the intensity of GFAP immunostaining was observed in samples from all neurodegenerative diseases in comparison with controls. All pathological cerebella shared similar findings concerning GFAP detection, an intense radial gliosis when GFAP intensity was higher (Fig. 4, upper row corresponding to GFAP) or matching varicose fibres parallel to the pial surface when GFAP intensity was lower (Fig. 4, lower row corresponding to GFAP). Astrogliosis seemed to be correlated with Purkinje cell loss in some occasions or at least with their damage.

Astrogliosis and microgliosis distribution are shown per layer in Fig. 5. One of the most noteworthy findings consisted of null semi-quantitative scores for microglial, in contrast to the highest for astroglial cell counts in the Purkinje cell layer.

Reactive microglia immunostaining was observed only sparsely in cerebella from prion-like disease-affected patients. The intensity and pattern were similar to those of the control patients. These glial cells mainly presented a ramified or dystrophic morphology (Fig. 4, row corresponding to microglia). Meanwhile, additional microglial shapes (rod-like and amoeboid, specifically) were found in the CJD cerebella. The extent of immunolabelling detecting reactive microglia was much higher in prion-affected cerebella than that detected in prion-like disease and control sections. *Post hoc* testing showed the existence of significant differences between them ($p < 0.05$). Microglial cells were extended to all layers in the former and were mostly located in white matter in the latter. Only in a few cases were they also present in the granular layer.

## 4. Discussion

To our knowledge, this is the first study to focus on the comparative assessment of glial profiles in human cerebella from affected prion and prion-like disorder patients. To suggest specific glial subpopulations in which prion diseases have an involvement similar to prion-like disorders was the final objective of the present study. A morphological approach using the cerebellum as an encephalic region scarcely studied in neurodegenerative diseases and a reference area in neuroinflammation research has been performed to this end.

Prion-like disorder is a new medical term referring to neurological proteinopathies with a common denominator: disease-causing proteins

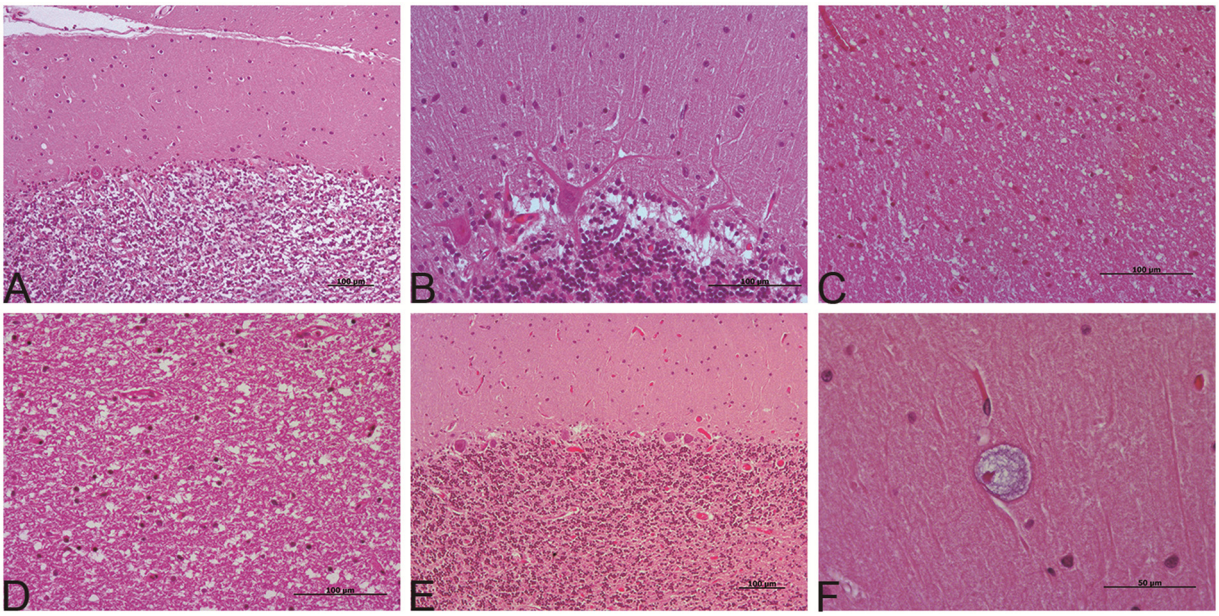


**Fig. 2.** Illustration of histopathological findings by optical microscopy (haematoxylin - eosin staining, HE) (A) Granular layer degeneration was observed in some cases, as shown here in a FTD specimen. (B) Decrease and swelling of Purkinje cells were very often observed, as shown in a PD specimen. (C) Typical vacuolation associated with prion disease is evidenced in a CJD specimen. (D) Vacuolation presenting an irregular morphology distinguishable from that typically found in CJD is shown in an FTD specimen. (E) No vacuolation was found in the control specimen. (F) Protein aggregates found in the molecular layer from a PD specimen.

spread in the brain in the same way as that first reported for prions (Prusiner, 2012; 2013; Fernandez-Borges et al., 2013). Despite the fact that, until recently, this common feature has been considered a leading factor in the neurodegenerative process that has been the main research focus, the pathogenic mechanisms are still unknown. Consequently, current therapeutic and palliative treatments are ineffective.

Historically, research regarding neurodegenerative disorders has focused on neurons as the main player in the neurodegenerative mechanism. However, more recent studies suggest glia as prominent contributors to this mechanism and even in some instances to disease initiation. The link between the neuroinflammation hypothesis and the main neurodegenerative disorder, such as AD (Rogers et al., 1992; Guerriero et al., 2017), has also been extended to other neurodegenerative diseases (Hirsch et al., 2012; Crotti and Glass, 2015; Radford et al., 2015). Alteration of the BBB (Zhao et al., 2015) and release of cytokines are characteristics of this neuroinflammatory process (Estes

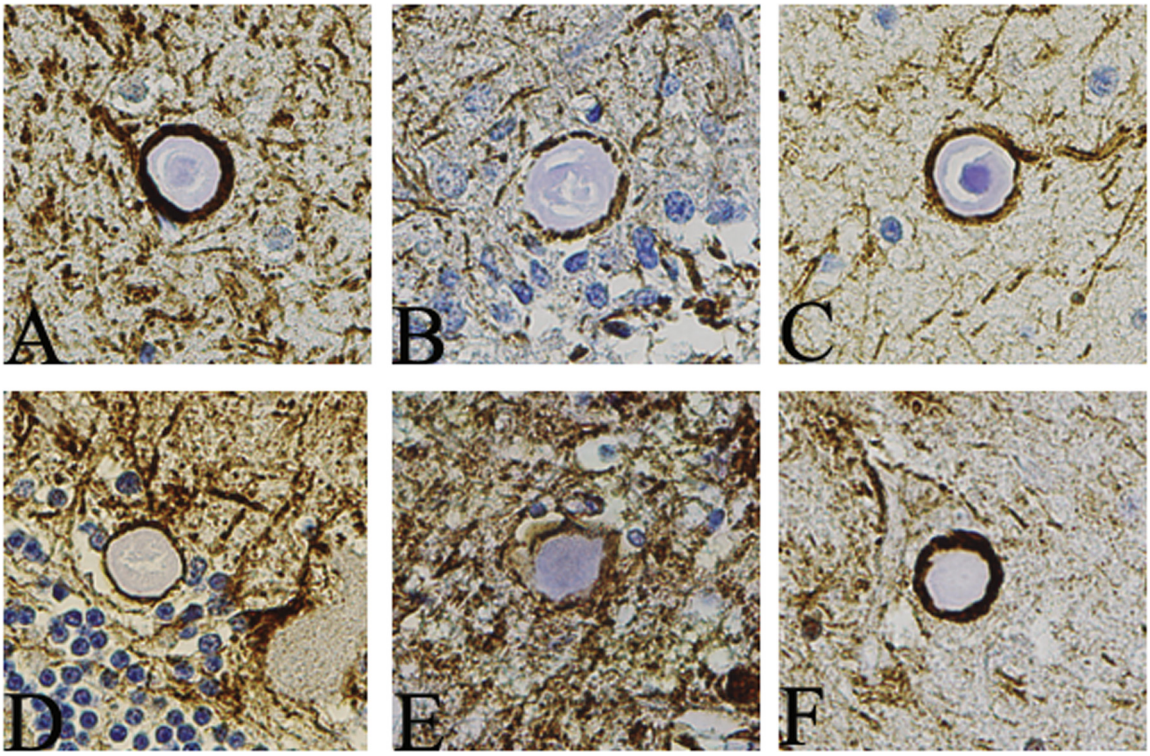


**Fig. 3.** Astroglial immunostaining was always detected surrounding protein aggregates in Purkinje cells or molecular layers from (A) AD, (B) PD, (C) HD, (D) FTD, (E) MND and (F) CJD-affected cerebella.

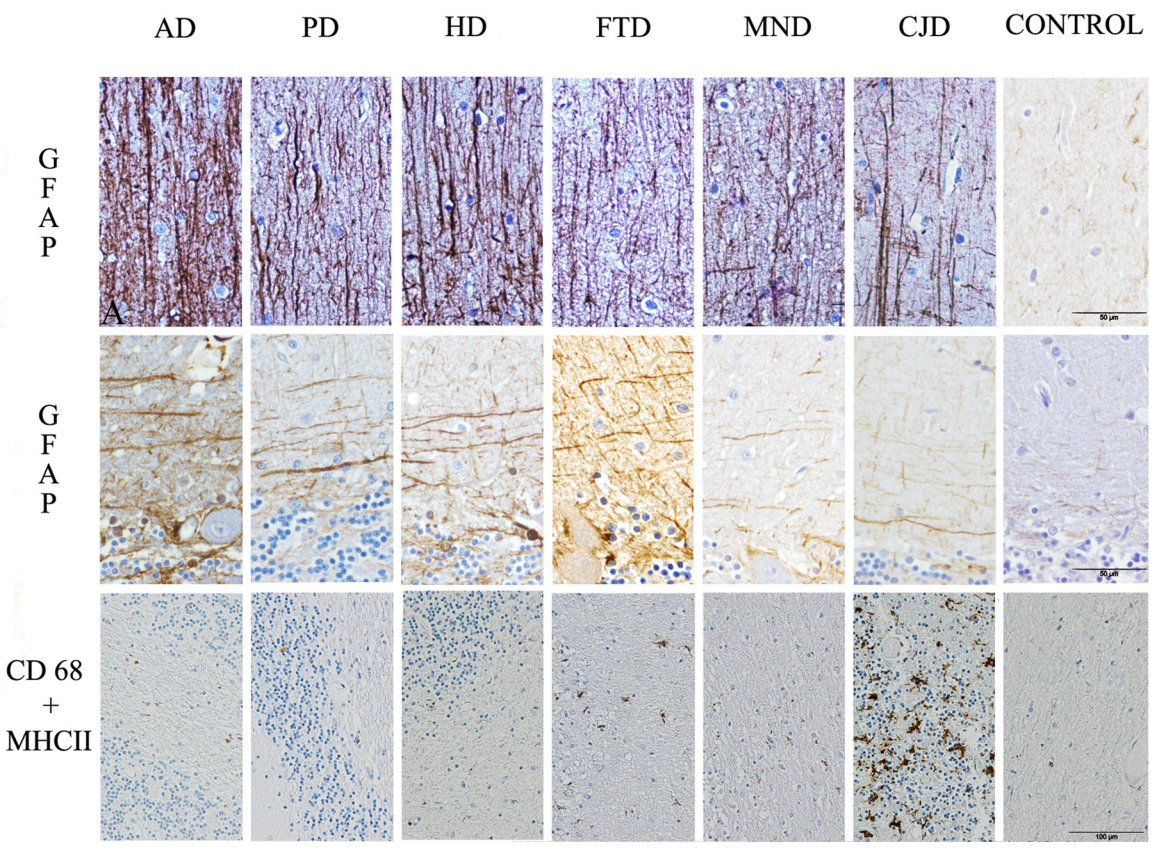


**Fig. 4.** Immunohistochemical pattern distribution observed when astroglial (GFAP) or reactive microglial (CD68 + MHC II) markers were assessed in sagittal cerebella corresponding to AD, PD, HD, FTD, MND and CJD as well as control specimens are shown.
Concerning GFAP immunostaining, a radial (upper row) or horizontal (parallel to the pial surface; lower row) profiles were consistently observed in the molecular layer. Astrogliosis and Purkinje cell damage are shown in close association.
With regards to CD68 and MHC II studies, sparse immunostaining (mostly ramified or dystrophic morphology) was mainly located in white matter in samples from AD, PD, HD, FTD, MND and control. However, immunolabelling was highly extended, even in the granular layer, in the CJD specimen.

and McAllister, 2014; DiSabato et al., 2016), and direct or indirectly, these features are related to glial cell population (Crotti and Glass, 2015).

In fact, microglial activation seems to be accepted as the main process leading to neuroinflammation (Derecki et al., 2014). Microglia constitute an immune network scanning any pathological changes in the microenvironment (Davalos et al., 2005; Nimmerjahn et al., 2005). Because of their enormous plasticity, they can change their morphology and show a broad spectrum of phenotypic appearances corresponding to different disease states (Kreutzberg, 1996; Streit et al., 2014; Bachstetter et al., 2015). This particular feature is in accordance with results provided here, where differences among microglial shapes depending on the pathology would suggest a higher involvement of this activated glial population in prion than in prion-like disorders. Ramified amoeboid morphologies of these glial cells extended throughout the prion-affected cerebella demonstrate their evident activation (Eitzen et al., 1998; Brown, 2001). As the amoeboid phenotype corresponds to the most activated shape (Boche et al., 2013b; Streit et al., 2014) and is associated with the phagocytic state, the presence of this phenotype in prion disease samples seems to be stimulated by the high accumulation of pathological prion protein (Muhleisen et al., 1995). This would also justify the absence of amoeboid microglia in prion-like disease-affected cerebella, as this brain area does not constitute the preferential site for the accumulation of the characteristic aberrant protein. Overall, the role of microglial activation in neurodegeneration may be related to their phagocytic properties for aberrant protein removal to a higher extent than with other functions attributed to this cellular type.

On the other hand, microglial cells showing dystrophic appearance may indicate neuropathological impairment. They are probably capable of releasing cytokines to stimulate the inflammatory brain response, presumably by astrocytes (Bianco et al., 2005; Saijo and Glass, 2011; Cekanaviciute and Buckwalter, 2016). In fact, it has been recently demonstrated that activated microglia can induce astrogliosis with neurotoxic functions (Liddelow et al., 2017b). The findings described in this study would be in accordance with this possible induction. However, the design of the study does not allow shed light on the role this early microglial activation is playing in neurodegenerative diseases. Further studies will be necessary in order to determine it.

Nevertheless, despite astroglial population remains only indirectly associated with neuroinflammation, our previous studies using natural Scrapie as a prion model demonstrated an indisputable relationship between this glial population and the propagation and evolution of the disease (Sarasa et al., 2012; Hernandez et al., 2014). The present study provided findings in the same line concerning not only prion but also other neurodegenerative disorders studied here. Astrocytic profiles similar to those found in the Scrapie model were shared by all of them. This confirms astrocyte activation as a common denominator for prion and prion-like disorders. The consistent presence of astroglial populations around protein deposits reinforces the involvement of these glial cells in neurodegenerative progress.

Those astroglial immunohistochemical patterns found in the cerebella from patients affected by all disorders included here were similar to those reported in the study dealing with natural Scrapie as a model of prion diseases. They matched up with Bergmann glia and varicose fibres parallel to the pial surface. Both probably originate in the

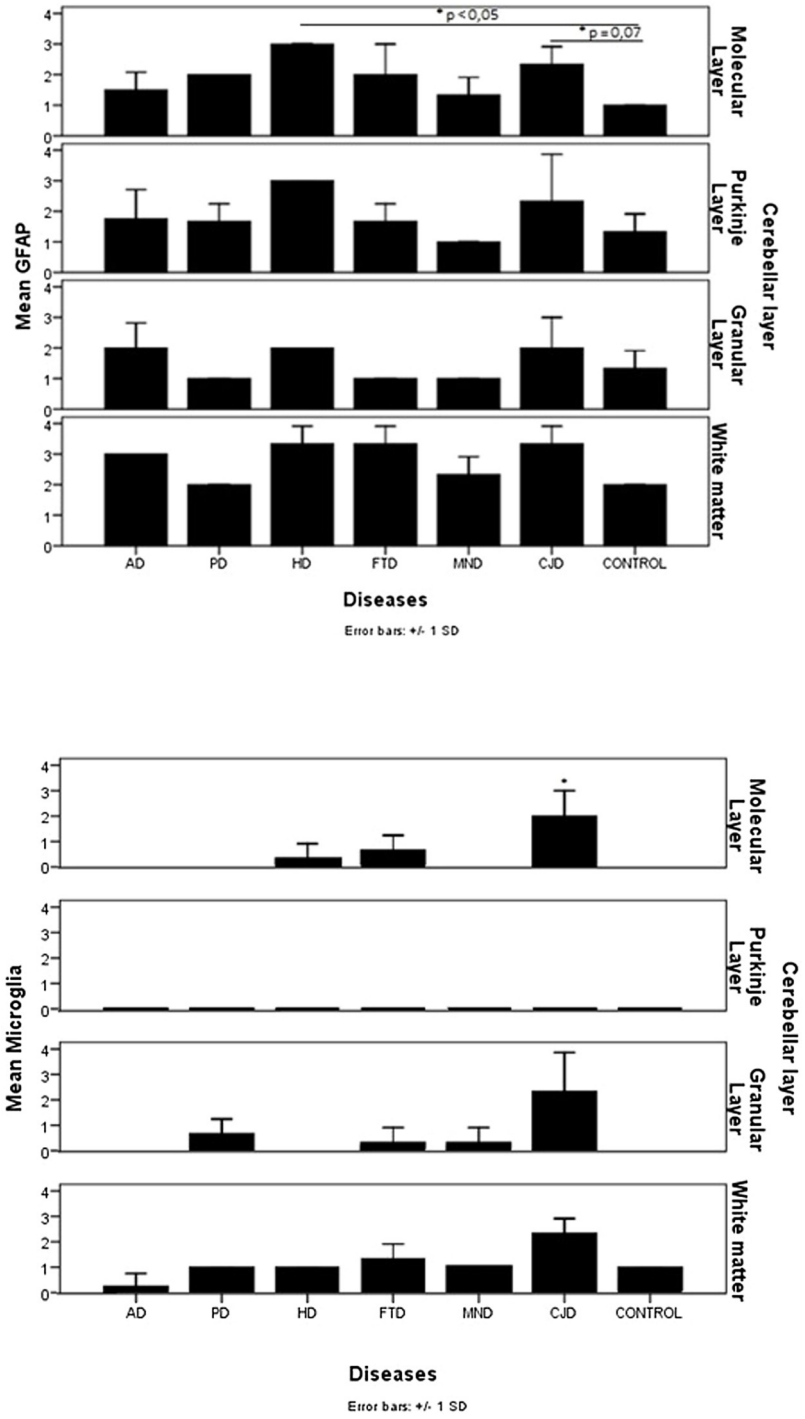


**Fig. 5.** Astroglia and microglia scores between groups (AD, PD, HD, FTD, MND, CJD and control) in different layers are compared. Y axes represent semi-quantitative scale mean, and bars represent ± SE; * $p < 0.05$, Kruskal-Wallis, Scheffe *post hoc*.

hypertrophic process of epithelial and stellate astroglia resident in Purkinje and granule cell layers, as well as in the generation of new astroglial cells in the hyperplasic process characteristic of astrogliosis. This suggests that the glial stem cell response would potentially protect against or compensate for neuronal loss (Alvarez et al., 2015). To determine whether they represent different states as neural progenitors for neuroglial cells, further studies focused on their role as stem cells are currently being developed. Some authors have postulated that variations in neurogenic process may play important roles in the alteration of neural stem cells differentiation into new born neurons during adult neurogenesis (Horgusluoglu et al., 2016).

On the other hand, the highest affectation of the Purkinje cell layer observed in the cerebellum from all samples assessed here has also been previously reported in relation to other neurodegenerative disorders (Sarna and Hawkes, 2003). A close relationship between an increase in hypertrophic astrocytes and loss of Purkinje cells in this layer has also been postulated in familiar (Fukutani et al., 1996) and sporadic AD (Garcés et al., 2016). Similar observations were also provided by the Scrapie model used by our group in the previously cited study (Hernandez et al., 2014). When they were closely observed in ultrastructural studies, vacuolation preferentially occurring around Purkinje cells displayed a close relation with glial cells (Sarasa et al., 2015). This

fact again points to astroglia as the main cellular type involved in neurodegenerative lesions.

Several hypotheses have been formed about the potential role of neuroglia in neuroinflammation, although they are still under discussion. These cells could exclusively have a neuroprotective role due to immune properties (Wyss-Coray et al., 2001; Liu et al., 2017). However, they could also imply a neurotoxic effect provoked by over-activation deriving from chronic inflammation (Streit et al., 2004; Heneka et al., 2015). They could also alter homeostasis, causing brain damage or functional loss.

In conclusion, the present study represents a novel advance regarding this subject because astroglial activation has been confirmed as a common cellular denominator for prion and prion-like disorders in the area assessed. The involvement of astrocytes as key elements in the pathogenesis and pathology of diseases and injuries of the central nervous system has been addressed in a very recently published study (Ferrer, 2017), suggesting that their knowledge will permit a better understanding of brain ageing and neurodegenerative diseases as complex disorders in which neurons are not the only players.

Moreover, similar immunohistochemical patterns for this astrocytic reaction found in the cerebella from patients affected by all neurodegenerative disorders suggest a glial stem cell response. Further studies are necessary to clarify their protective or neurotoxic role in neurodegenerative progression.

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