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Short communication

Autophagy impairment in highly prion-affected brain areas of sheep experimentally infected with atypical scrapie

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ABSTRACT

Autophagy is a critical physiologic process contributing to the maintenance of cell homeostasis. Autophagy dysfunction has been directly linked to a growing number of neurodegenerative disorders, including prion diseases. However, little is known about the molecular mechanisms underlying autophagic failure and its connection with prion neuropathology. In a previous work we described alterations of this process in the central nervous system (CNS) of sheep naturally infected with classical scrapie, although specific neuronal populations such as Purkinje cells seemed to display an autophagy-related neuroprotective effect against prion toxicity. As atypical scrapie displays a lesion pattern different to the one observed in the classical form, using immunohistochemical analyses, we further investigated herein the role of autophagy in the CNS of sheep experimentally infected with atypical scrapie prions. While ATG5 protein showed a similar distribution in atypical scrapie to that observed in the classical form, expression of LC3-B and LC3-A did not change in any brain region. However, p62, a marker of impaired autophagy, was overexpressed in the most prion-affected areas, including Purkinje cells, which suggests that autophagic activity is deteriorated in the CNS of atypical scrapie and these cells are also susceptible to neurotoxicity and do not exhibit a general defensive mechanism based on autophagy. By comparing data from both clinical scrapie forms, we have demonstrated that autophagy impairment is highly dependent on the neuropathological lesion levels of the brain area analysed and may be implicated in prion neuropathology.

1. Introduction

Autophagy, an intracellular process involved in protein and organelle turnover by lysosome degradation, has been associated with several pathologies, including neurodegenerative diseases (Larsen and Sulzer, 2002). The presence of autophagic vacuoles has been described in experimental models of TSEs, in induced scrapie and in the natural disease in humans (Heiseke et al., 2010). Nevertheless, even though autophagy has been repeatedly proposed to provide a protective effect in neurons (Yao et al., 2013), the precise connection of this process with TSEs neuropathology, or even whether autophagy is completely beneficial or pathogenic during neurodegeneration, is still unclear.

In a previous study we described a decrease of autophagy in highly-affected areas of the central nervous system (CNS) in classical scrapie that could facilitate prion replication (Lopez-Perez et al., 2019). This decrease manifested as a downregulation of autophagy-related genes and an increment of ATG5 and p62 autophagy markers. In contrast, the correlation observed between autophagy markers and prion deposition, and the intense immunoreactivity displayed by LC3 markers, mainly in Purkinje cells, suggested the induction of this process as a defence mechanism in specific cell populations.

To confirm this assumption and the association between prion-related lesions and autophagy arrest, we investigated the distribution of autophagy markers in the CNS of sheep experimentally infected with atypical scrapie, considering that atypical and classical forms of scrapie differ in their neuropathological and molecular phenotypes (Arsac et al., 2007; Benestad et al., 2008). This paper describes the overexpression of p62, but not LC3 proteins, in highly-affected areas of atypical scrapie brains, including Purkinje cells, confirming the association between prion-related lesions and autophagy downregulation in a different form of the disease.

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2. Materials and methods

2.1. Animals

A panel of 4 female sheep (Churra Tensina breed) experimentally infected with atypical scrapie by intracerebral inoculation were used in this study. Isolate consisted of CNS material from a Spanish sheep naturally affected with atypical scrapie (AHQ/AHQ) elaborated and supplied by Centre de Recerca en Sanitat Animal (CReSA) of Barcelona, Spain. The methodology used for inoculation is detailed elsewhere (Marín et al., 2018). All of them were sacrificed when showing clear clinical symptoms, including 1 animal in the terminal stage of the disease. Atypical scrapie animals were adults of age 46.08 ± 4.19 months carrying the AHQ/AHQ genotype for *PRNP* (prion protein) gene, which is the most frequently affected genotype in atypical scrapie animals (Benestad et al., 2008). In order to compare our findings with those obtained in the classical form of the disease, neuropathological and immunohistochemical results were compared to those obtained in 6 negative scrapie-free sheep used as controls in our previous work (Lopez-Perez et al., 2019). All of them were female of Rasa Aragonesa breed, with ARQ/ARQ genotype and age 48.81 ± 16.93 months.

2.2. Ethics statement

The Ethics Committee for Animal Experiments of the University of Zaragoza (Permit Number: PI13/10) approved all procedures. The care and use of experimental animals were performed in strict accordance with the national law (R.D. 53/2013).

2.3. Histopathology, PrP^{Sc} deposition and immunohistochemical determination of autophagy-related proteins

Formalin-fixed samples of frontal cortex (Fc), basal ganglia (Bg), basal ganglia cortex (Bgc), thalamic cortex (Tc), thalamus (T), pons (P), cerebellum (Cbl) and medulla oblongata (Mo) were trimmed and processed according to standard procedures. For histopathology, 4-μm-thick tissue sections were deparaffinized and stained with haematoxylin and eosin (HE). Confirmatory immunohistochemistry (IHC) and PrP^{Sc} distribution profiling were performed in adjacent sections using mouse monoclonal antibody 8G8 [1:200 dilution at room temperature (RT) for 30 min; Cayman Chemical, Michigan, USA], as previously reported (Andreoletti et al., 2011). Scrapie negative and positive control tissue slides from confirmed cases were included in each IHC run as internal technical controls.

Autophagy markers ATG5, LC3-B, LC3-A and p62 were determined by IHC using the following primary antibodies: mouse monoclonal anti APG5 (C-1, sc-133158; Santa Cruz Biotechnology), mouse monoclonal anti MAP-LC3β (G-2, sc-271625; Santa Cruz Biotechnology), rabbit polyclonal anti MAP-LC3α (R-23, sc-134226; Santa Cruz Biotechnology) and rabbit polyclonal anti p62 (PW9860; Enzo Life Sciences). The methodology used was previously described in our study performed in classical scrapie (Lopez-Perez et al., 2019). To ensure specificity of the IHC reactions we included in each run a background control section for nonspecific staining, in which the primary antibody was omitted, and a classical scrapie section from our previous study for specific staining.

2.4. Data analysis

Spongiform changes (i.e. neuropil spongiosis and intraneuronal vacuolation), intensity of PrP^{Sc} staining, as well as immunolabelling of autophagy markers, were examined with a ZEISS Axioskop 40 optical microscope and scored semi-quantitatively on a scale ranging from 0 (absent) to 5 (severe) (Supplementary Fig. 1) in the eight aforementioned brain regions. In addition, four layers of the Cbl [molecular layer (Ml), Purkinje layer (Pl), granular layer (Gl) and white matter (Wm)], and five neuronal nuclei of the Mo [the hypoglossal motor nucleus (HMN), the dorsal nucleus of the vagus nerve (NVN), the lateral cuneate nucleus (LCN), the nucleus of the trigeminal nerve spinal tract (NTN) and the olivary nucleus (ON)] were also analysed in order to obtain a more detailed description of these areas. Scores obtained in controls from our previous study (Lopez-Perez et al., 2019) were used to compare classical and atypical results. In both studies, the same pathologists evaluated each brain region using a 10x magnification and moving through the entire tissue. The final score for each brain region was calculated as the mean of the layers or nuclei analysed. A higher magnification (40x) was used to describe and identify the location of staining. The reliability of this subjective quantification was confirmed by Western blot in our previous work (Lopez-Perez et al., 2019). Histopathological and immunohistochemical differences with controls were evaluated using non-parametric Mann Whitney U test. Correlations between protein immunolabelling and the different neuropathological lesions in atypical scrapie were determined using the non-parametric Spearman´s rank correlation coefficient (rho, ρ). IBM® SPSS® statistics 22 software was used for all data analysis. The results were considered significant at $P < 0.05$.

3. Results

3.1. Prion deposition and related lesions

The pattern of neuropathological lesions observed in the brain samples corresponded to that described for the atypical form of scrapie (Benestad et al., 2008; Nentwig et al., 2007). To obtain this pattern, scores were compared to those obtained in negative scrapie-free sheep used as controls in our previous work (Lopez-Perez et al., 2019).

The evaluation of HE-stained sections revealed a significant and strong increase of neuropil spongiosis in Fc, Bg, Bgc, Tc and Cbl of the scrapie-infected animals compared with controls (Fig. 1A). In contrast, this pathological lesion was much less prominent, but still significant, in T, P and Mo. Intraneuronal vacuolation displayed a similar distribution pattern, with a small but significant increment in Fc, Bg, Bgc, Tc and Cbl (Fig. 1B). In this case, vacuolation was totally absent in T, P and Mo.

IHC for PrP^{Sc} confirmed the diagnosis of scrapie, and positivity was detected in all animals studied. However, the intensity of PrP^{Sc} deposits was markedly different between the scrapie affected animals, showing one animal minimal scores in almost all CNS areas analysed, except for Cbl, another sheep displayed moderate PrP^{Sc} immunolabelling, and two of them showed severe staining intensity (data not shown). Despite the high variability observed, the Fc, Bg, Bgc, Tc and Cbl showed the highest scores for PrP^{Sc} deposition (Fig. 1C).

When analysing the cerebellar layers separately, we observed a significant and strong increment of spongiosis (Fig. 1A) and PrP^{Sc} immunostaining (Fig. 1C) in Ml, Gl and Wm, which did not show vacuolation (Fig. 1B). In contrast, the Purkinje layer displayed no PrP^{Sc} deposition (Fig. 1C), but great intensity of spongiosis and vacuolation (Fig. 1A and B), although the latter was highly variable within the animals. In Mo, minimal changes were observed in the different neuronal nuclei for the studied lesions (Fig. 1A-C). Fig. 2A and B show representative images demonstrating the presence of scrapie-associated pathology in cerebellum.

3.2. Distribution of autophagy markers in the CNS of atypical scrapie animals

The immunostaining patterns of autophagy markers were identical to those previously described for classical scrapie (Lopez-Perez et al., 2019). IHC revealed an upregulation of ATG5 in the Mo of atypical scrapie-infected animals compared to the control group ($P = 0.033$), specifically in the NTN ($P = 0.028$), but also in the HMN ($P = 0.033$) and the NVN ($P = 0.011$) (Figs. 3A and 4 A), whereas there were no significant differences in the remaining tissues under study. Scores for

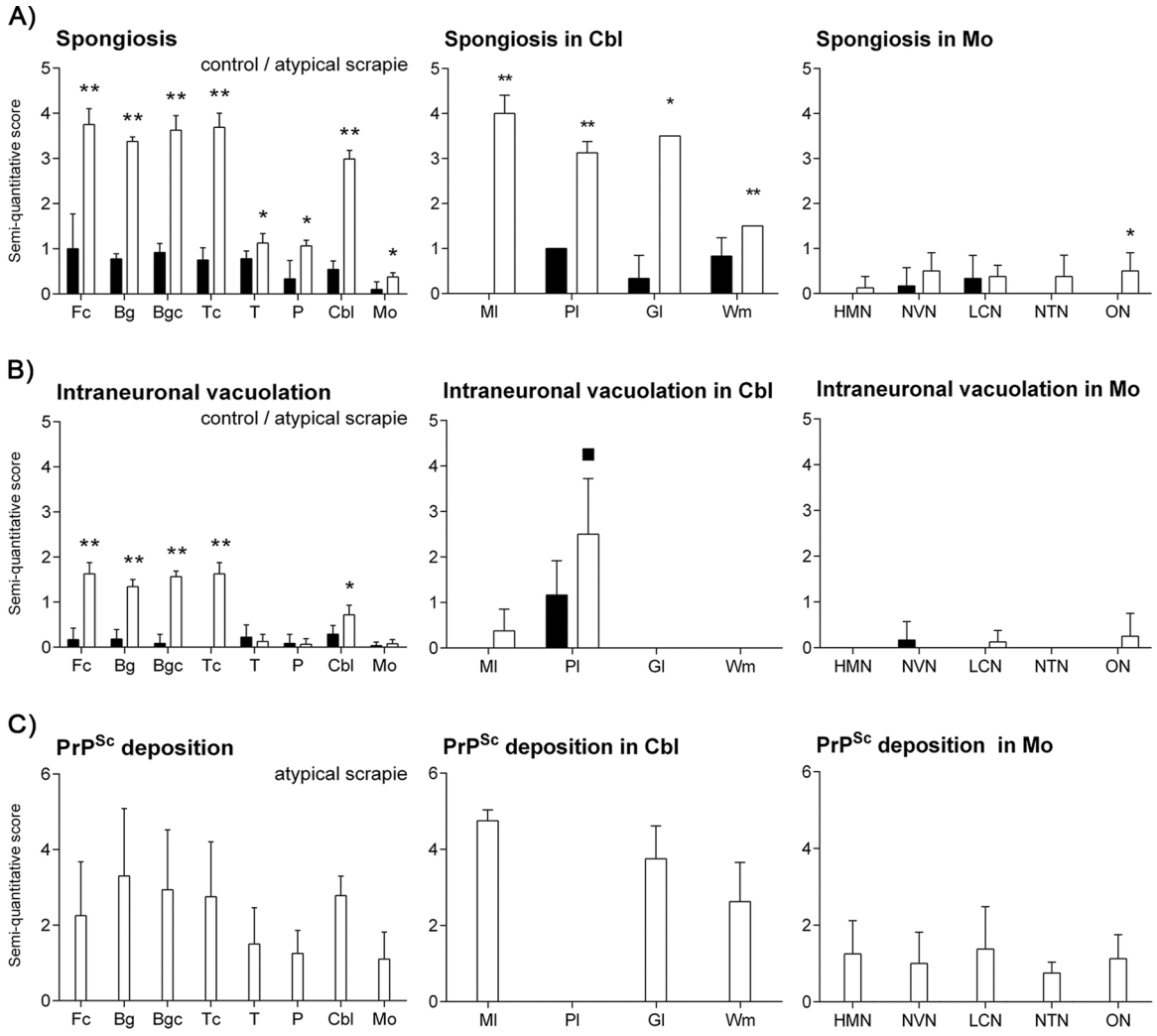

Fig. 1. Semi-quantitative scoring of neuropil spongiosis (A), intraneuronal vacuolation (B) and PrP^{Sc} deposition (C) in different brain areas of atypical scrapie-infected sheep (white bars) and control animals (black bars). Graphics show the semi-quantitative assessment values of neuropathological features in frontal cortex (Fc), basal ganglia (Bg), basal ganglia cortex (Bgc), thalamic cortex (Tc), thalamus (T), pons (P), cerebellum (Cbl) and medulla oblongata (Mo), including four cerebellar layers [molecular layer (Ml), Purkinje layer (Pl), granular layer (Gl) and white matter (Wm)], and five neuronal nuclei of the Mo [the hypoglossal motor nucleus (HMN), the dorsal nucleus of the vagus nerve (NVN), the lateral cuneate nucleus (LCN), the nucleus of the trigeminal nerve spinal tract (NTN) and the olivary nucleus (ON)]. Scores range from 0 (negative) to 5 (maximum intensity). Significant differences were determined using the Mann Whitney U test (■$P < 0.1$, *$P < 0.05$ and **$P < 0.01$).

p62, the impaired-autophagy marker, were significantly higher in highly-affected brain areas like Fc ($P = 0.015$), Bg ($P = 0.031$), Bgc ($P = 0.038$), Tc ($P = 0.014$), and Cbl ($P = 0.035$), including all cerebellar layers (Figs. 3B and 4 B and C). Interestingly, the minimally prion-affected regions in these animals, i.e. T, P and Mo, showed no significant changes of p62 levels (Fig. 3B).

Most of the vacuolised and dysmorphic Purkinje cells in the cerebella of atypical scrapie sheep did not show ATG5 staining, or it was of a lower degree, compared to those cells apparently healthy (Fig. 4D), which led to a tendency to downregulation of this marker in this cerebellar area ($P = 0.052$) (Fig. 3A). This finding was also observed for both LC3-B and LC3-A (Fig. 4E), although these proteins did not show semi-quantitative differences neither in Cbl nor the rest of brain tissues (Fig. 3C and D). Unlike the other markers, p62 staining levels were similar within the Purkinje cell layer, regardless of their cellular integrity or shape (Fig. 4C).

ATG5 and p62 immunostaining scores correlated with all neuropathological features of the atypical scrapie group ($P < 0.001$), but in the opposite way (negatively for ATG5 and positively for p62) (Table 1). LC3-A positively correlated with neuropil spongiosis ($P < 0.01$) and intraneuronal vacuolation ($P < 0.001$), and LC3-B showed no significant correlations.

4. Discussion

Abnormal or perturbed autophagic activity is frequently observed in several neurodegenerative diseases (Son et al., 2012; Wong and Cuervo, 2010). In prion diseases, autophagy seems to be a host defence response to infection that plays a protective role by degrading the pathological PrP^{Sc} accumulated within neurons *in vitro* (Aguib et al., 2009; Heiseke et al., 2010), and autophagy dysfunction in affected neurons may contribute to the formation of spongiform changes (Yao et al., 2013). We described a possible impairment of autophagy in some of the most highly-affected brain regions of sheep naturally affected with classical scrapie, whereas mechanisms compatible with induction of autophagy were observed in other areas, suggesting a combination of anti-prion and prion-promoting effects throughout the brain during the course of the disease (Lopez-Perez et al., 2019). Since atypical scrapie differs from the classical form, among other properties, in the neuroanatomical distribution of the histopathological lesions and PrP^{Sc} deposits in the

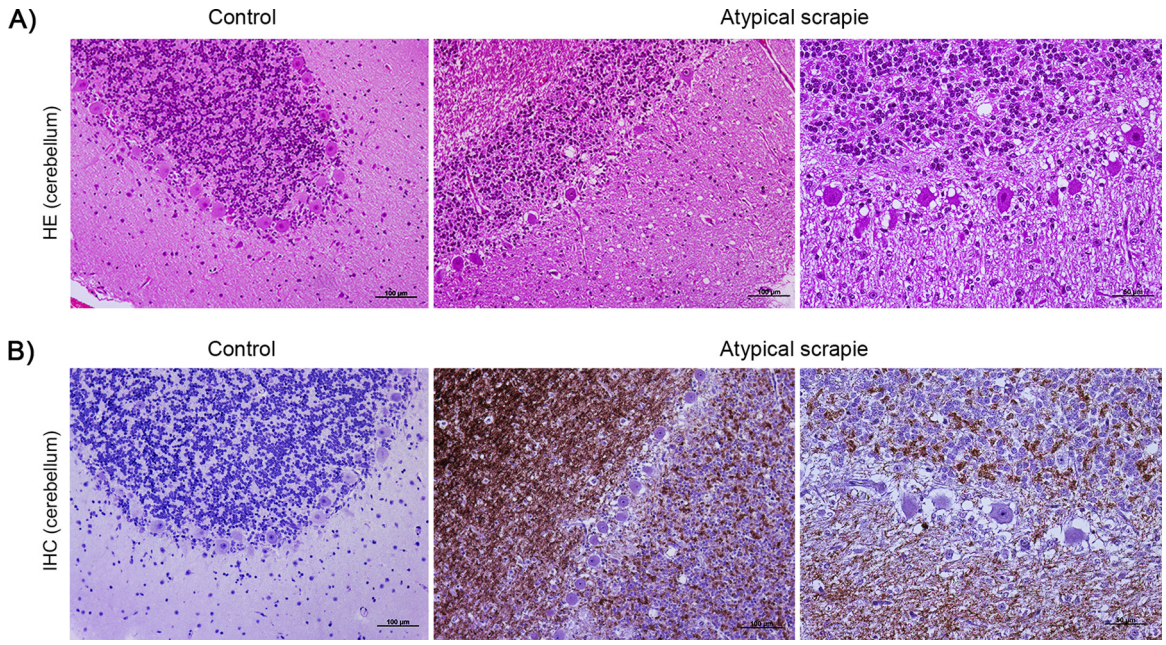

Fig. 2. Histopathological and immunohistochemical features of atypical scrapie-infected sheep and control animals. Figure shows representative images of (A) haematoxylin and eosin staining (HE), and (B) immunohistochemistry (IHC) for PrP^{Sc} deposition in cerebellum of control and atypical scrapie sheep (100 and 50 μm).

brain (Benestad et al., 2008), we investigated here if both strains display similar autophagy responses. In this study, the neuropathological profile of experimentally inoculated sheep corresponded to that described for atypical scrapie (Benestad et al., 2008; Nentwig et al., 2007). Infected sheep displayed high variability in PrP^{Sc} deposition. The neuropathological diversity in this disease is frequently observed among the affected individuals, not finding a clear correlation between this heterogeneity and factors such as the *PRNP* genotype (Nentwig et al., 2007). It has been suggested that atypical scrapie is caused by a uniform kind of prion, rather than by different strains, and unknown host factors could contribute to the variability in the neuroanatomical distribution of PrP^{Sc} (Gotte et al., 2011).

ATG5, a protein that plays a crucial role in phagophore expansion and autophagosome development (Romanov et al., 2012), showed a similar immunohistochemical distribution in atypical scrapie to that observed in the classical form (Lopez-Perez et al., 2019), including an upregulation in Mo. Unlike classical scrapie, this brain area displays a low degree of lesion in the atypical disorder, thus ATG5 upregulation is not related to the accumulation of PrP^{Sc}, and indeed it displayed a negative correlation with scrapie lesions. This early marker is not present in the mature vesicle after its complete formation (Mizushima et al., 2003, 2001), consequently, it is not involved in the subsequent steps of autophagic machinery. Therefore, ATG5 may be important for the initiation of the process, but may not reflect the resulting degradative event.

LC3 proteins are considered to be a hallmark for autophagy and are widely used for monitoring this process (Mizushima and Yoshimori, 2007). The accumulation of p62, a cargo receptor subjected to autophagic clearance via autophagosome degradation (Myeku and Figueiredo-Pereira, 2011; Pankiv et al., 2007), is commonly used as a marker to detect impairment or defects in autophagic activity (Bjorkoy et al., 2005, 2009). Then, the comparative evaluation of LC3 and p62 accumulation is required to verify an autophagic response (Jeong and Park, 2015; Niklaus et al., 2017).

In classical scrapie, LC3-B increased in Bg and Cbl and LC3-A in specific neuronal populations of Mo, P and Cbl (Lopez-Perez et al., 2019). On the contrary, the expression of both LC3 isoforms did not show significant changes in any brain area of atypical scrapie animals when compared to the control group, suggesting that the amount of autophagosomes in infected animals is the same as that in healthy ones. In addition, whereas p62 increased in a generalized manner throughout the CNS in the classical form of the disease (Lopez-Perez et al., 2019), this protein was significantly overexpressed only in the most prion-affected areas (Fc, Bg, Bgc, Tc and Cbl) of atypical scrapie and their immunostaining scores positively correlated with all neuropathological features. Accumulation of p62 in all highly-affected brain areas would indicate that autophagic activity is deteriorated in the CNS of atypical scrapie, which suggests that, even with the same levels of autophagosomes as the control group, those of the healthy ones are more functional and effective than those of scrapie animals, since they can successfully clear p62. A primary defect in the ability of autophagosomes to recognize cytosolic cargo was identified in cellular and mouse models of Huntington´s disease (Martinez-Vicente et al., 2010). Autophagosomes formed at normal rates and were adequately eliminated by lysosomes, but they failed to efficiently trap cytosolic cargo in their lumen, leading to the accumulation of the mutant huntingtin protein in cells, which resembles our specific scenario. The inefficient engulfment of PrP^{Sc} by non-functional autophagosomes could be responsible for the accumulation of this protein in highly-affected CNS areas in atypical scrapie.

However, impairment of autophagy does not lead to PrP^{Sc} accumulation in all types of neuronal cells. So far, intraneuronal staining pattern of PrP^{Sc} has not been described in atypical scrapie (Benestad et al., 2008; Moore et al., 2008). Cbl is one of the tissues that shows remarkable neuropathological differences between classical and atypical scrapie. Overexpression of LC3-A and p62 proteins in almost all Purkinje cells in classical scrapie, suggested an activation of autophagy as a defence mechanism against neurodegeneration, since these cells are minimally affected in this scrapie form (Lopez-Perez et al., 2019). However, atypical scrapie brains showed a discernible absence of ATG5, LC3-B and LC3-A staining in a great amount of apparently degenerating Purkinje cells, while healthy cells in the same tissue remained stained. Moreover, we could not appreciate staining differences of p62 in Purkinje cells, which displayed similar p62 staining levels whether or not they were damaged and a significant overexpression compared to healthy animals. Overexpression of p62 in damaged cells,

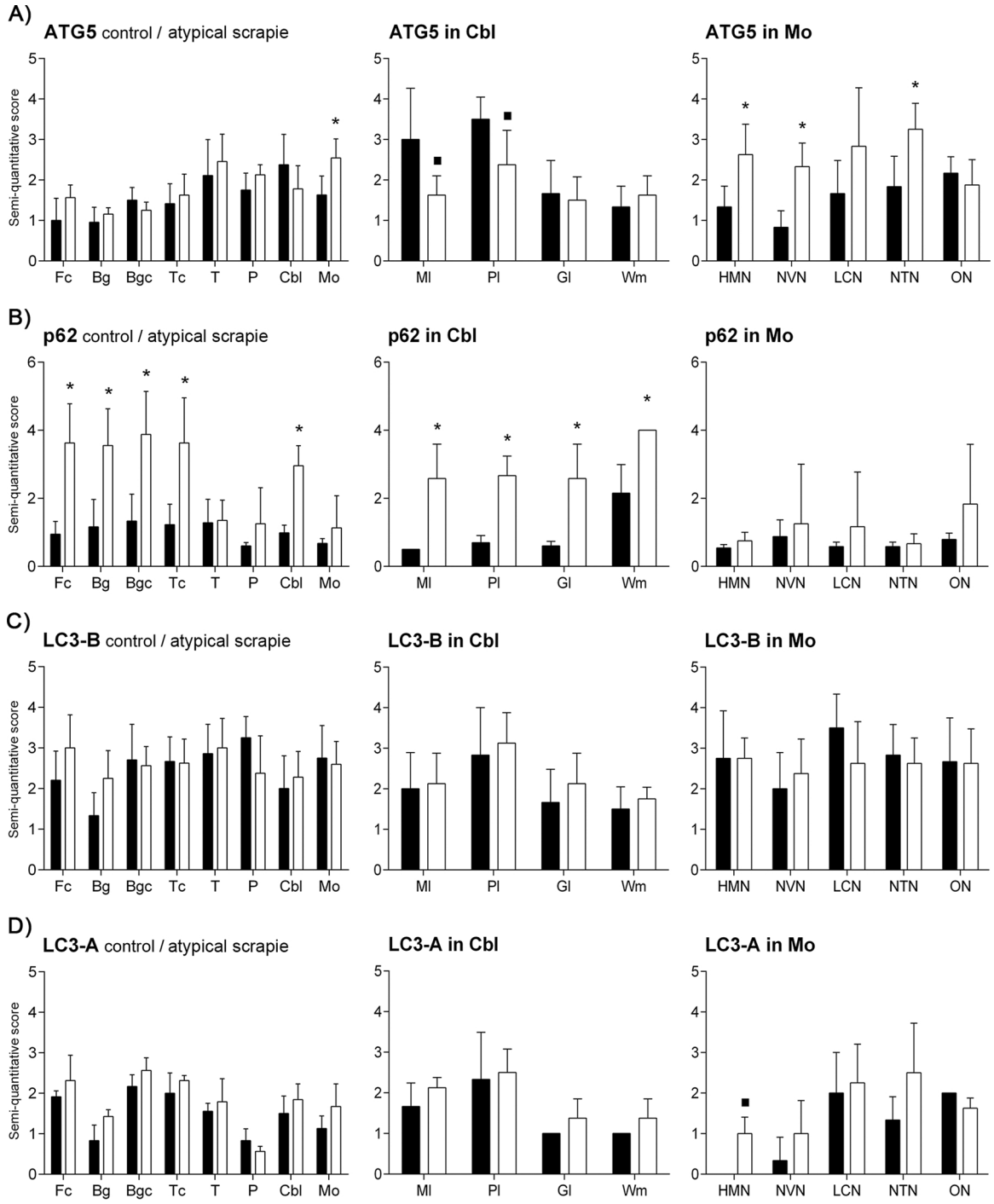

Fig. 3. Semi-quantitative scoring of immunohistochemistry for autophagy-related proteins in atypical scrapie-infected sheep (white bars) compared to control animals (black bars). Score values of (A) ATG5, (B) p62, (C) LC3-B and (D) LC3-A (from 0: negative, to 5: staining present at its maximum intensity) evaluated in frontal cortex (Fc), basal ganglia (Bg), basal ganglia cortex (Bgc), thalamic cortex (Tc), thalamus (T), pons (P), four layers of cerebellum (Cbl) [molecular layer (Ml), Purkinje layer (Pl), granular layer (Gl) and white matter (Wm)], and five neuronal nuclei of the medulla oblongata (Mo) [the hypoglossal motor nucleus (HMN), the dorsal nucleus of the vagus nerve (NVN), the lateral cuneate nucleus (LCN), the nucleus of the trigeminal nerve spinal tract (NTN) and the olivary nucleus (ON)]. The differences between the two experimental groups were determined using the Mann Whitney U test (■$P < 0.1$ and *$P < 0.05$).

but not LC3 or ATG5, suggests that autophagy may be perturbed during the neurodegenerative process, which could lead to the pathological phenotype in these cells but not to PrP^{Sc} accumulation. The differences observed between classical and atypical prions suggest that both strains alter the autophagic process, although via different molecular mechanisms.

The lack of autophagy induction in slightly injured areas (T, P and Mo), the positive correlation between p62 and prion-related lesions and the absence of PrP^{Sc} in Purkinje cells in spite of the decrease of autophagy suggest that dysfunction of autophagic machinery could be a consequence of prion toxicity rather than the cause of prion accumulation in atypical scrapie. More research will be necessary to confirm the particular role of autophagy under prion infection, involving different animal models and clinical stages, which will improve understanding of pathogenic mechanisms of dysfunctional autophagy and help develop therapeutic strategies for prion diseases.

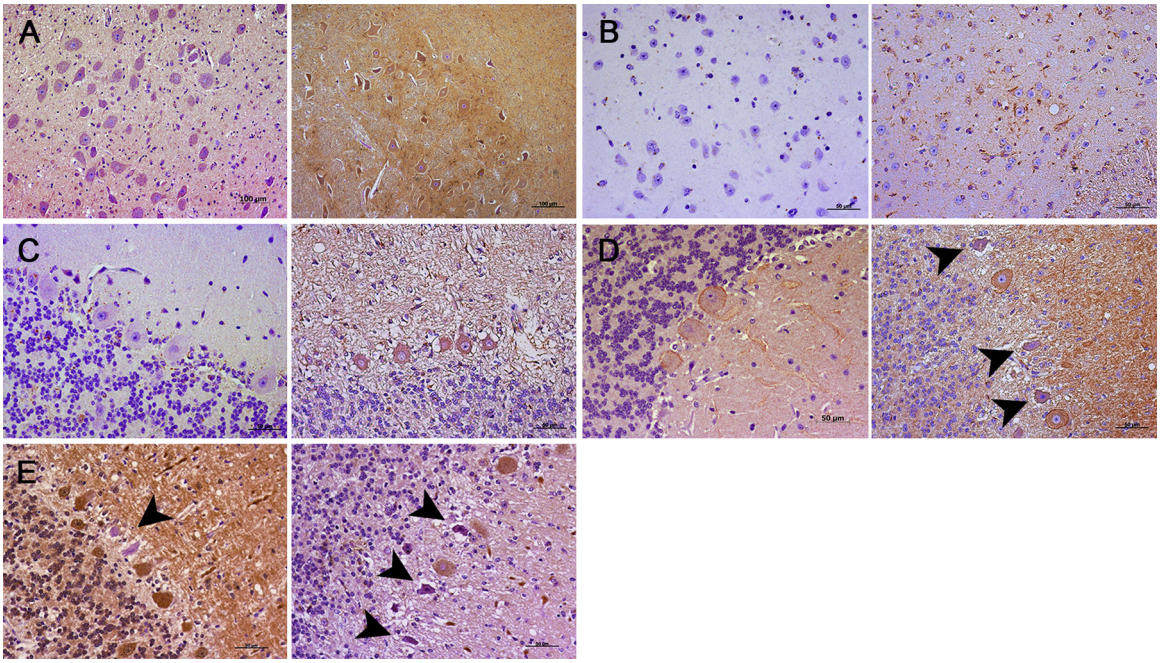

Fig. 4. Immunostaining patterns of autophagy-related proteins in different CNS regions of atypical scrapie sheep compared to control animals. (A) Intense immunostaining of ATG5 in the dorsal nucleus of the vagus nerve in atypical scrapie animals (right; score: 3), and weak staining in control animals (left; score: 1) (100 μm). (B) Strong immunolabelling of p62 in caudate nucleus of basal ganglia in atypical scrapie sheep (right; score: 4), and weak staining in control animals (left; score: 1) (50 μm). (C) Overexpression of p62 in cerebellum of atypical scrapie animals (right; mean score: 3.75) compared to the control group (left; mean score: 0.75). p62 levels were similar within the Purkinje cells in the atypical scrapie group, regardless of their cellular integrity (right) (50 μm). (D) Similar immunostaining of ATG5 in cerebellum of atypical scrapie animals (right; mean score: 2.25) and control sheep (left; mean score: 2.5). In atypical scrapie, those Purkinje cells in poor condition did not show ATG5 staining, or it was of a lower degree (arrowheads) (50 μm). (E) Absence of both LC3-B (left) and LC3-A (right) staining in degenerating Purkinje cells of cerebellum (arrowheads) from atypical scrapie sheep (50 μm).

Table 1
Spearman correlation values between autophagy markers scores and histological features in atypical scrapie infected sheep.

	Scrapie lesions			Autophagy markers		
Autophagy markers	Spongiosis	Intraneuronal vacuolation	PrP^{Sc}	LC3-A	LC3-B	p62
ATG5	−0.469***	−0.358***	−0.240*	N.S.	0.449***	−0.390***
LC3-A	0.297**	0.388***	N.S.	—	0.317**	N.S.
LC3-B	N.S.	N.S.	N.S.	0.317**	—	N.S.
p62	0.635***	0.552***	0.670***	N.S.	N.S.	—

Correlations were estimated using data from the eight analysed tissues.
No statistically significant correlation values are shown as N.S.
(*P < 0.05, **P < 0.01 and ***P < 0.001).

5. Conclusion

Autophagy dysregulation certainly does occur in both classical and atypical scrapie. By comparing both clinical forms, we have demonstrated that autophagic impairment is a prominent feature of highly-affected brain areas, suggesting that this process is involved in prion-induced toxicity. It is very likely that autophagy may be implicated in TSEs neuropathology and, even though this process may be activated, there are substantial signs indicating an impairment or failure in the degradative event, which could contribute to the pathogenesis of the disease.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.04.026.

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