Received: 9 October 2019 | Accepted: 19 December 2019

DOI: 10.1002/jcp.29426

ORIGINAL RESEARCH ARTICLE

Journal of Cellular Physiology WILEY

Autophagy and NLRP3 inflammasome crosstalk in neuroinflammation in aged bovine brains

Davide De Biase[1] | Giuseppe Piegari[1] | Francesco Prisco[1] | Ilaria Cimmino[2] | Claudio Pirozzi[3] | Giuseppina Mattace Raso[3] | Francesco Oriente[2] | Edoardo Grieco[4] | Serenella Papparella[1] | Orlando Paciello[1]

[1]Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Naples, Italy

[2]Department of Translational Medicine, University of Naples "Federico II", Naples, Italy

[3]Department of Pharmacy, University of Naples "Federico II", Naples, Italy

[4]Azienda Sanitaria Locale, Salerno, Italy

Correspondence
Davide De Biase, Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Via Federico Delpino 1, 80137, Naples, Italy.
Email: davide.debiase@unina.it

Abstract

NLRP3 inflammasome is a multiprotein complex that can sense several stimuli such as autophagy dysregulation and increased reactive oxygen species production stimulating inflammation by priming the maturation of proinflammatory cytokines interleukin-1β and interleukin-18 in their active form. In the aging brain, these cytokines can mediate the innate immunity response priming microglial activation. Here, we describe the results of immunohistochemical and molecular analysis carried out on bovine brains. Our results support the hypothesis that the age-related impairment in cellular housekeeping mechanisms and the increased oxidative stress can trigger the inflammatory danger sensor NLRP3. Moreover, according to the recent scientific literature, we demonstrate the presence of an age-related proinflammatory environment in aged brains consisting in an upregulation of interleukin-1β, an increased microglial activation and increased NLRP3 expression. Finally, we suggest that bovine may potentially be a pivotal animal model for brain aging studies.

KEYWORDS

aging, autophagy, bovine, immunosenescence, neuroinflammation, NLRP3 inflammasome

1 | INTRODUCTION

"Immunosenescence" is one of the most recognized effects of aging and consists in the dysregulation of the immune system as a result of defects in both initiation and resolution of immune responses (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). Immunosenescence is accompanied by a low-grade and chronic proinflammatory environment in multiple tissues characterized by increased production of proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), acute-phase proteins, reactive oxygen species (ROS), and autoantibodies. This proinflammatory environment has been defined as "inflammaging" and it has been linked to an increased incidence of several disorders, including neurodegenerative diseases (Franceschi et al., 2007; Montecino-Rodriguez, Berent-Maoz, & Dorshkind, 2013). Microglia, the innate immune cells of the central nervous system (CNS), most likely contribute to the development of this age-related proinflammatory environment and it is speculated that any changes in microglial activities are key components in influencing the pathogenesis of neurodegeneration (Luo, Ding, & Chen, 2010). Microglia normally keep in a quiescent state when not challenged, but rapidly transform to an activated state when triggered by appropriate stimulation showing a deramified shape and enhanced expression of activation-associated markers such as major histocompatibility complex II (MHC II; Sheffield & Berman, 1998). An emerging growing body of evidence in animal models sustains that inflammatory responses involving microglia and interleukin-1β (IL-1β) secretion play a pivotal role in the progression of diseases of the CNS (Glass, Saijo, Winner, Marchetto, & Gage, 2010). The molecular steps leading to IL-1β maturation take place in an intracellular complex known as "inflammasome" (Martinon, Burns, & Tschopp, 2002). The inflammasome is a caspase-1-activating multiprotein platform that results from

oligomerization of inactive monomeric proteins from the nucleotide-binding domain, leucine-rich repeat (NLR) protein family (Martinon et al., 2002). Although there are several subfamilies of NLRs and different complexes have been described, the most intensively studied is the NLRP3 inflammasome which is formed when NLRP3 associates with the adapter protein "Apoptosis-associated speck-like protein containing a CARD" (ASC) and procaspase-1 upon activation by different stimuli, such as pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), or endogenous danger signals such as increased ROS (Latz, Xiao, & Stutz, 2013; Walsh, Muruve, & Power, 2014). The precise mechanism of NLRP3 inflammasome activation is not fully understood, but it is thought that its activation occurs probably not through direct ligand–receptor interaction but rather by the sensing of cellular homeostasis disruption (Ratsimandresey, Dorfleutner, & Stehlik, 2013; Wen, Miao, & Ting, 2013). Autophagy, specifically macroautophagy, is an intracellular homeostatic mechanism for the sequestration and degradation/recycling of cytosolic components (Batatinha, Diniz, de Souza Teixeira, Krüger, & Rosa-Neto, 2019; Pagano et al., 2015; Pascarella et al., 2018). Several lines of evidence suggest that autophagy and inflammasomes mediate dynamic crosstalk between the two systems to maintain homeostasis during stress conditions inside cells (Harris et al., 2017; Yuk & Jo, 2013). With these premises, the present study aimed to corroborate the presence of an age-related proinflammatory environment in bovine brains by evaluating the immunohistochemical and pattern expression of MHC II and NLRP3 inflammasome and the molecular analysis of IL-1β and interleukin-18 (IL-18) level in brain tissue.

Based on the hypothesis that the age-related impairment in cellular housekeeping mechanisms and the increased oxidative stress can trigger the inflammatory danger sensor NLRP3, a further objective was to study the colocalization and the potential association between NLRP3 inflammasome, increased ROS production, and autophagy dysregulation in brain aging.

2 | MATERIALS AND METHODS

2.1 | Animals

For this study, morphological, immunohistochemical, and molecular analysis were carried out on 42 brain samples collected from Podolic dairy cattle (1–24 years old). Animals were divided in three groups: Group A (aged) included bovine aged 15–24 years ($n = 14$), Group B (adult) included bovine aged 5–14 years ($n = 14$), and Group C (young) included bovine aged up to 5 years ($n = 14$).

Inclusion criteria for animal selection comprised a thorough physical examination by which any apparent clinical illness or neurological sign (gait abnormalities, weakness, and decreased mental status) were excluded. Moreover, the absence of prion diseases was confirmed in all animals by performing the rapid test recommended by European law. All sampling procedures from animals were performed during post-mortem inspection in an abattoir in Campania Region, Italy; thus, the study did not require consent or ethical approval according to European Directive 2010/63/EU. However, the animals were slaughtered in strict accordance with European slaughter regulations (CE no: 1099/2009 of September 24, 2009) for the protection of animals at the time of killing (Directive, 2009). Permission to obtain the samples was granted from the owner of the abattoir and from the veterinary inspector responsible for the sanitary surveillance.

At the slaughterhouse, the brain was immediately removed and fixed for 15 days in 10% neutral buffered formalin for histological and immunohistochemical examination. Samples of hippocampus were also collected and frozen at −80°C until further processing. For the present study, transversal sections were taken from superior frontal gyrus and hippocampus (dentate gyrus). Precisely, for hippocampal formation, we analyzed pyramidal cells layer, Cornu ammonis (CA2, CA3, and CA4) fields. Sections were subsequently embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (HE) for morphology and periodic acid–Schiff (PAS) for lipofuscin (De Biase & Paciello, 2015).

2.2 | Immunohistochemistry (IHC)

IHC experiments were performed to evaluate the expression of NLRP3 and MHC II. For IHC, 4-μm-thick sections of hippocampus were mounted on positively charged glass slides (Bio-Optica, Milan). For antibody detection, we used a well-established protocol described elsewhere (De Biase et al., 2018). Briefly, antigen retrieval pretreatments were performed using a HIER citrate buffer pH 6.0 (Bio-Optica, Milan, Italy) for 20 min at 98°C. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (H_2O_2) in methanol and sections were blocked with a protein block (MACH1; Biocare Medical LLC, Concord, CA) for 30 min each. Slides were sequentially incubated overnight at 4°C with primary rabbit polyclonal NLRP3 antibody (LS-B8262; LifeSpan BioSciences) diluted 1:400 in phosphate-buffered saline (PBS 0.01 M, pH 7.2) and mouse monoclonal MHC II antibody diluted 1:200 in PBS (clone H42A; VMRD). Antibody deposition was visualized using the 3,3′-diaminobenzidine (DAB) chromogen diluted in DAB substrate buffer and the slides were counterstained with hematoxylin. Between all incubation steps, slides were washed two times (5 min each) in PBS. In the corresponding negative control sections, the primary antibody was either omitted or replaced with normal serum from the same species of primary antibody (rabbit). Sections from an intestinal biopsy of human patients affected by Crohn's disease, known to display the expression of NLRP3 antibody, were used as a positive control.

2.3 | Double-color immunofluorescence

Double immunofluorescence was used to qualitatively investigate NLRP3-Beclin1 and NLRP3-SOD1 coexpression in bovine brains. The pretreatment steps were the same as those used for immunoperoxidase labeling and the procedure was described elsewhere (De Biase et al., 2018). Primary antibodies used in this study included rabbit

polyclonal Beclin1 (BECN 1 H300: sc-11,427; Santa Cruz Biotechnology, Dallas, TX), diluted 1:400 in PBS (0.01 M PBS, pH 7.2) and rabbit polyclonal superoxide dismutase 1 (SOD1, ab13498; AbCam, Cambridge, UK) diluted 1:400 in PBS. Primary antibodies were applied overnight at 4°C. Slides were washed three times, 5 min each, in PBS and incubated for 2 hr at room temperature with a Goat anti-rabbit IgG (H + L) Secondary Antibody (Alexa Fluor® 546 conjugate, A-11035; Thermo Fisher Scientific, Rockford, IL) diluted 1 in 100 in PBS, for 2 hr at room temperature. A further protein blocking step was performed by incubating slides with normal rabbit serum (Vector Laboratories) for 15 min at room temperature, before the overnight application of the second primary antibody. For NLRP3 detection, after three washes in PBS, a rabbit polyclonal anti-NLRP3 antibody (LS-B8262; LifeSpan BioSciences) diluted 1:400, was applied and the sections were again incubated overnight at 4°C. Slides were washed again three times in PBS and then incubated with a fluorescein isothiocyanate (FITC)-conjugated pure goat anti-rabbit secondary antibody (Alexa Fluor® 488 conjugate, A-11034; Thermo Fisher Scientific) diluted 1 in 100 in PBS, for 2 hr at room temperature. Slides were rinsed with PBS and mounted with a solution of one part glycerol per one part PBS. For scanning and photography, a laser scanning microscope (LSM 510; Zeiss, Göttingen, Germany) was used. The anti-NLRP3 polyclonal rabbit antibody bound to FITC was illuminated at 488 nm and read using a 505–560 nm bandpass filter. Rabbit polyclonal anti-BECN1 and anti-SOD1 antibodies bound to tetramethylrhodamine isothiocyanate (TRITC) were illuminated at 543 nm and then read with a 560 nm long-pass filter.

As for immunohistochemistry, a negative control was performed in all instances by omitting the primary antibody and incubating tissue sections with PBS and/or replacing it by an irrelevant antibody. The different frames were scanned separately, with appropriate installation of the optical path for excitation and emission of each scan according to the manufacturer's instructions.

For immunohistochemistry and immunofluorescence analysis, cytoplasmic MHC II, NLRP3, Beclin1, and SOD1 immunolabeling was assessed semiquantitatively by two independent pathologists (O. P. and D. D. B.) under blinded conditions, with a concordance rate of 95%. Immunolabeling was measured for each antibody in 10 randomly selected high-powered fields (×40) as the percentage of positively stained cells referred to the entire studied sections and graded as it follows: 0, absent; 1 (mild), >1% to <25% of cells; 2 (moderate), 26% to <50%; 3 (high), >50%.

2.4 | Western blot analysis

Samples of brain from elder cows and controls were cut at the cryostat at 20 μm and then lysed at 4°C in 200 μl of TBS lysis buffer (Tris-buffered saline, 20 mM Tris-HCl pH 7.6, 140 mM NaCl, 30 mM sodium pyrophosphate, 5 mM ethylenediaminetetraacetic acid, 0.55% nonidet P40, 1% Triton X-100, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM iodoacetamide, 1 mM phenanthroline). Protein concentration in the supernatant was determined by bicinchoninic acid assay (BCA) protein assay (BCA: Pierce Biotechnology, Rockford, IL), and lysates were adjusted to equivalent concentrations with lysis buffer. Aliquots of 10 mg of total brain lysate were then separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride membranes that were blocked overnight at 4°C with 5% nonfat dried skimmed milk in TTBS (TBS with 0.05% Tween 20). Incubation with primary specific antibodies against NLRP3 (1:1,000 dilution) and horseradish peroxidase-conjugated secondary antibodies was performed in blocking solution for 1 hr at room temperature. Immunoreactive bands were visualized by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology). The same blots were stripped and reprobed using anti-GAPDH monoclonal antibody to confirm equal loading of proteins in all lanes. Band intensities were quantified on scanned images using Image J software (National Institute of Health) to determine average pixel intensity.

2.5 | Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis

A real-time PCR analysis was performed to evaluate the changes of IL-1β and IL-18 levels in brain. Total cellular RNA was isolated from bovine brain samples by using the Rneasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. One microgram of cell RNA was reverse-transcribed using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA). PCR reactions were analyzed using iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA). Reactions were performed using Platinum SYBR Green qPCR SuperMix-UDG using an iCycler iQ multicolor Real-Time PCR Detection System (Bio-Rad; Cimmino et al., 2019). All reactions were performed in triplicate and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Primer sequences used are described in Table 1.

2.6 | Statistical analysis

Analyses were performed with GraphPad (version 5.03; GraphPad Software Inc., CA). Statistical comparisons were made for each antibody by age group. A one-way analysis of variance was

TABLE 1 Primer sequences used in real-time RT-PCR analysis

Primers	Sequences
bIL-1β	Forward: 5′-TCCGACGAGTTTCTGTGTGA-3′ Reverse: 5′-ATACCCAAGGCCACAGGAAT-3′
bIL-18	Forward: 5′-TGGCAAACTTGAACCTAAGCT-3′ Reverse: 5′-TGAACAGTCAGAATCAGGCAT-3′
bGAPDH	Forward: 5′-TGGTGAAGGTCGGAGTGAA-3′ Reverse: 5′-GAAGGGGTCATTGATGGCGA-3′

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction.

performed to compare the overall level of MHC class II antigen and NLRP3 inflammasome labeling among age groups. The post-hoc tests employed were *t* tests for two samples assuming unequal variances. Data obtained from western blot analysis and RT-PCR were analyzed with Statview software (Abacus Concepts) by Student's *t* test. Blots were revealed by enhanced chemiluminescence and autoradiography using β-actin as a loading control. The autoradiographs shown are representative of four independent experiments. Bars represent the mean ± *SD* (standard deviation) of four independent experiments.

For all experiments, $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Morphology and immunohistochemistry

The morphological findings were similar to those previously described (De Biase et al., 2017; De Biase & Paciello, 2015) and revealed mostly moderate to severe satellitosis and gliosis and severe neuronal PAS-positive lipofuscin accumulation in mature and aged brains. No relevant pathologic changes were observed in the brains of young animals. Immunohistochemistry was performed to show the expression and distribution of the NLRP3 and MHC II antibody in the brains of young, mature, and aged bovine. MHC class II staining pattern was localized to endothelial cells and microglial cells that displayed a ramified morphology (Figure 1a–c). The prevalence of MHC II-positive microglial cells in studied groups was as follows: Group A (aged): 80% high and 20% moderate; Group B (adult), 15% high, 85% moderate, and 5% mild; Group C (young), 95% negative and 5% mild. There was a statistically significant positive association between age and the presence of MHC II-labeled microglial cells ($p < .0001$; Figure 1d).

NLRP3 immunoreactivity was nearly absent in young animals (Group C; Figure 2a). Conversely, NLRP3 immunoreactivity was detected in aged and adult animals (Groups A and B) as brownish intracytoplasmic granules mostly in neurons and microglial cells (Figure 2b,c). Similar to MHC II expression, there was a statistically significant positive association between age and the presence of NLRP3-labeled cells ($p < .0001$; Figure 2d). The prevalence of NLRP3-positive cells in the studied groups was as follows: Group A (aged): 90% high and 10% moderate; Group B (adult): 30% high, 65% moderate, and 5% mild; Group C (young): 95% negative and 5% mild.

3.2 | Double-color immunofluorescence

Furthermore, we performed double-color immunofluorescence to evaluate the possible association between NLRP3 expression, autophagy, and increased ROS production. From a qualitative evaluation of the immunofluorescence-stained slides, the overall expression of Beclin1 as a marker for autophagy was similar to what we previously reported in a recent work (De Biase et al., 2017). TRITC-conjugated Beclin1 expression appeared as a red, cytoplasmic diffuse fluorescence and it was significantly decreased in aged and mature animals (95% mild and 5% moderate), whereas younger animals showed a higher expression (90% high and 10% moderate). TRITC-conjugated SOD1 expression appeared as a red, cytoplasmic, diffuse fluorescence. As expected, SOD1 overall expression was high or moderate (65% and 35%, respectively) in aged and mature animals but was absent or mild (<2%) in young animals. NLRP3

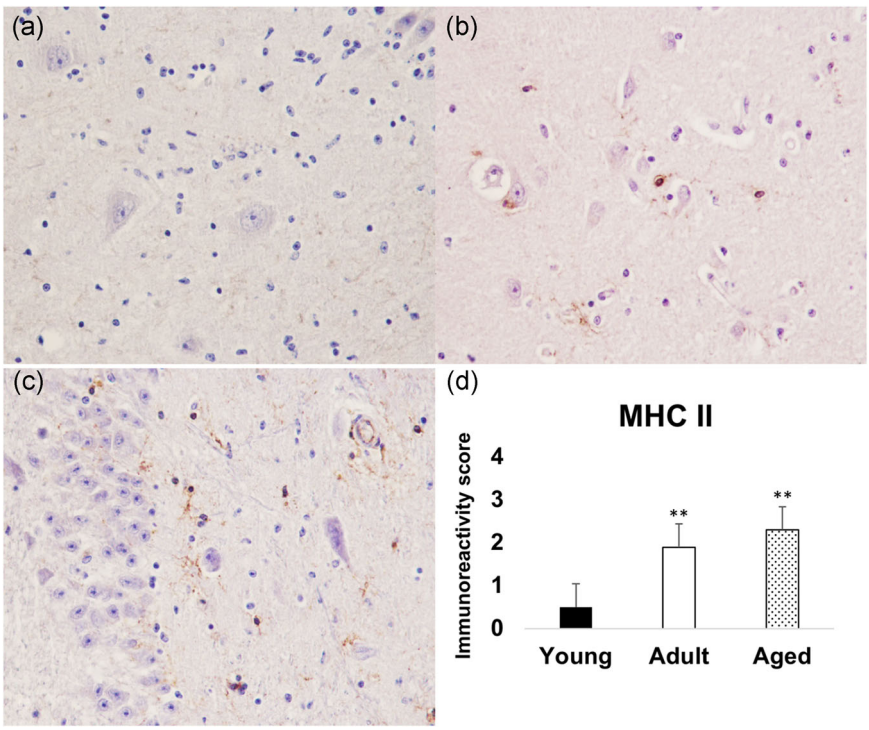

FIGURE 1 Immunohistochemical expression of MHC II, hippocampus, dentate gyrus in cows. (a) Young, (b) adult, and (c) aged animals. (d) Immunoreactivity score for MHC II expression. Original magnification, ×40. There is a statistically significant positive association between age and the presence of MHC II-labeled microglial cells. Each value is the mean ± *SEM* (**$p < .05$ vs. control). MHC, major histocompatibility complex; *SEM*, standard error of the mean

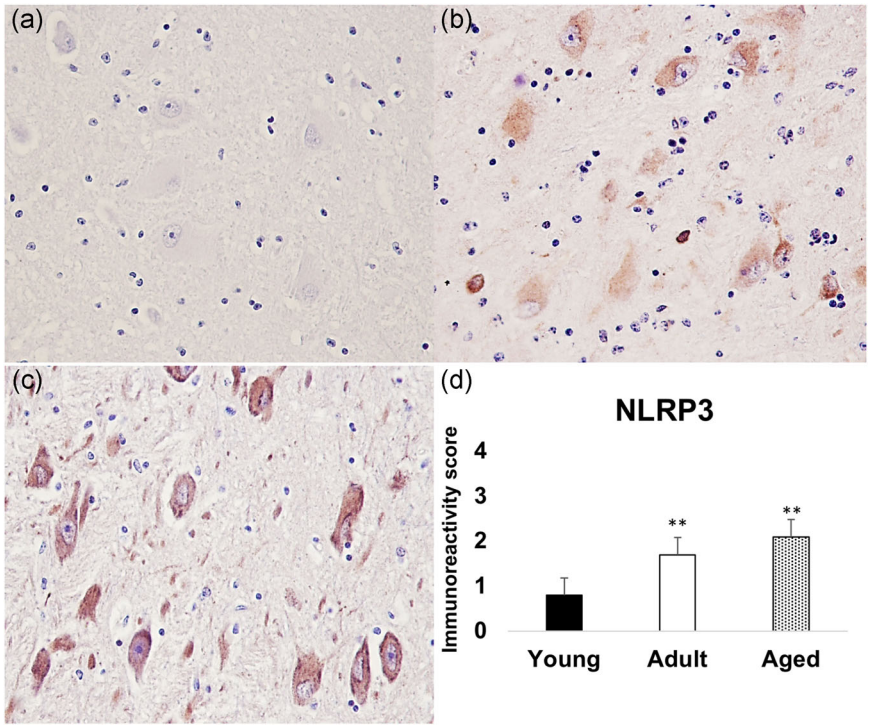

FIGURE 2 Immunohistochemical expression of NLRP3, hippocampus, dentate gyrus in cows. (a) Young, (b) adult, and (c) aged animals. (d) Immunoreactivity score for NLRP3 expression. Original magnification, ×40. There is a statistically significant positive association between age and the presence of NLRP3 immunolabeled cells. Each value is the mean ± *SEM* (**$p < .05$ vs. control). *SEM*, standard error of the mean

FITC-conjugated antibody was evident as a green, intracytoplasmic diffuse fluorescence. Colocalization of NLRP3 with either Beclin1 or SOD1 was evident as a strong, cytoplasmic diffuse, orange fluorescence (Figure 3a,b).

In aged and mature animals, NLRP3 + /Beclin1-cells were more numerous (85%) than NLRP3 + /Beclin1 + cells (15% of double-positive cells). Conversely, NLRP3+/SOD1+ cells were more in number (> 90%) than NLRP3+/SOD1− cells (<10%). In young animals, colocalization of NLRP3 with either Beclin1 or SOD1 was rarely observed (<2%) as NLRP3 and SOD1 expression was absent to mild (Figure 3c).

The inevitable age-related lipofuscin accumulation in neurons resulting in autofluorescence was considered in the qualitative assessment of primary antibodies expression. In our cases, lipofuscin accumulation was evident as a granular, yellow fluorescence usually localized at the periphery of the cytoplasm. However, the detection of the specific exogenous marker emission signal was not hindered by lipofuscin.

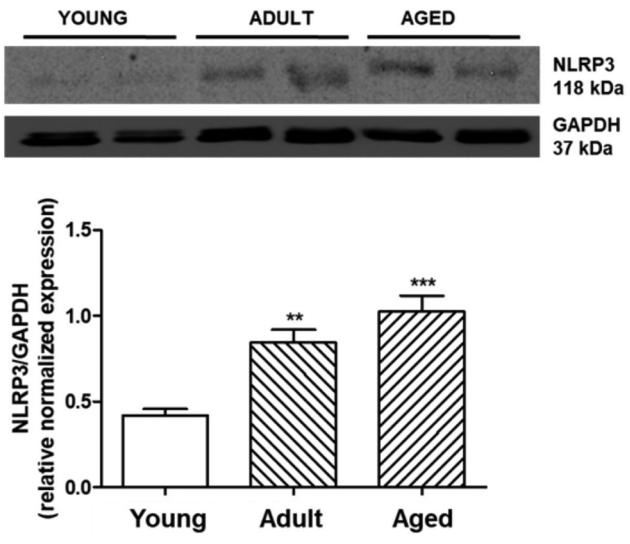

FIGURE 3 Western blot analysis for NLRP3 expression. (a, b) NLRP3 expression is significantly higher in the aged and adult animals (Groups A and B) compared with the young animals (Group B; **$p < .01$ vs. control) (Group C; ***$p < .001$ vs. control)

3.3 | Western blot analysis

We evaluated the expression levels of NLRP3 (represented as 118 kDa band) mainly to confirm an increased expression in brains from elder cows compared with young animals. As expected, our results, normalized for GAPDH, showed that NLRP3 expression was significantly higher in the aged animals (Groups A and B) compared with the young animals (Group C; $p < .001$ vs. control; Figure 4a,b).

3.4 | Real-time RT-PCR analysis

Changes of IL-1β and IL-18 levels in brain were measured with RT-PCR analysis. Our results showed that IL-18 and IL-1β were differently expressed in the three groups. IL-1β levels were increasingly higher from young to mature to aged animals ($p < .006$ vs. control). Surprisingly, IL-18 levels were higher in mature animals compared with young and aged ($p < .005$ vs. control). Moreover, IL-18 levels in young animals were slightly higher compared with aged (Figure 5a,b).

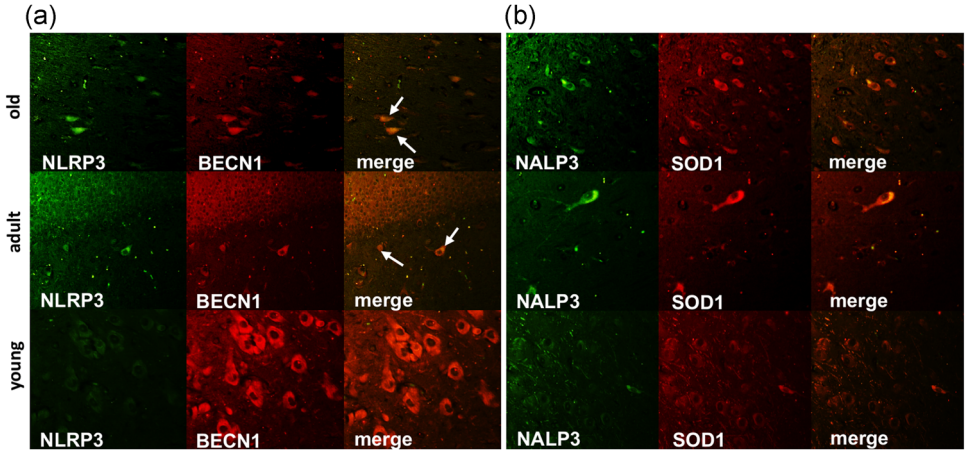

FIGURE 4 Double-color immunofluorescence of dentate gyrus, hippocampus in cow. (a) Green FITC immunofluorescence corresponds to NLRP3. Red TRITC immunofluorescence corresponds to Beclin1 labeling. Yellow fluorescence indicates colocalization of NLRP3 and Beclin1 (arrows). (b) Green FITC immunofluorescence corresponds to NLRP3. Red TRITC immunofluorescence corresponds to SOD1 labeling. Yellow fluorescence indicates colocalization of NLRP3 and SOD1. FITC, fluorescein isothiocyanate; SOD, superoxide dismutase; TRITC, tetramethylrhodamine isothiocyanate

4 | DISCUSSION

The term *neuroinflammation* is currently used to describe the inflammatory response originated in the CNS after suffering an injury (Morales, Guzmán-Martínez, Cerda-Troncoso, Farías, & Maccioni, 2014; Mortezaee, Khanlarkhani, Beyer, & Zendedel, 2018). Neuroinflammation is characterized by an increase in microglial activation, oxidative stress, and by the upregulation of inflammatory cytokines such as IL-1β and IL-18 (Deleidi, Jäggle, & Rubino, 2015; Figure 6). Although neuroinflammation plays an important role in the pathogenesis of many neurodegenerative diseases (Deleidi et al., 2015), it is widely accepted that neuroinflammatory changes also occur with age (Ward, Dexter, & Crichton, 2015).

A recent growing body of evidence has suggested an instrumental role of inflammasomes in the pathophysiology of neuroinflammation during neuronal aging (Singhal, Jaehne, Corrigan, Toben, & Baune, 2014).

Among the inflammasomes, NLRP3 is by far the most studied complex for its prominent role in several neurological diseases and also for its involvement in sterile inflammatory responses, antimicrobial responses, and hereditary autoinflammatory syndromes (Bauernfeind et al., 2011; Zhong, Sanchez-Lopez, & Karin, 2016). To date, most studies have focused on NLRP3 function in microglia, although NLRP3 has also been proposed to function in neurons (Compan et al., 2012; Ramos et al., 2012; von Herrmann et al., 2018). To our knowledge, this is the first study that investigates and describes the expression of NLRP3 inflammasome in bovine brains. Our results showed an increased expression of NLRP3 inflammasome in neurons and microglial cells of aged brains when compared with younger brains. These data are consistent with the recent scientific literature, indicating that the NLRP3 inflammasome is upregulated in the brain as a result of aging (Singhal et al., 2014).

It has been demonstrated that NLRP3 inflammasome activation follows two signals: transcription and post-translational modifications of

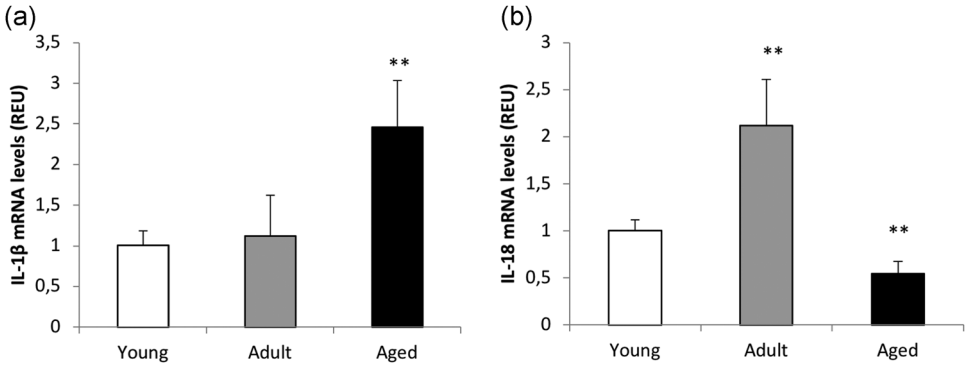

FIGURE 5 Changes of IL-1β and IL-18 levels in the brain were measured with RT-PCR analysis. Our results showed that IL-18 and IL-1β were differently expressed in the three groups. (a) IL-1β levels were increasingly higher from young to adult to aged animals (**$p < .006$ vs. control). (b) IL-18 levels were significantly higher in mature animals compared with young and aged (**$p < .005$ vs. control). Moreover, IL-18 levels in young animals were slightly higher compared with aged. RT-PCR, reverse-transcription polymerase chain reaction

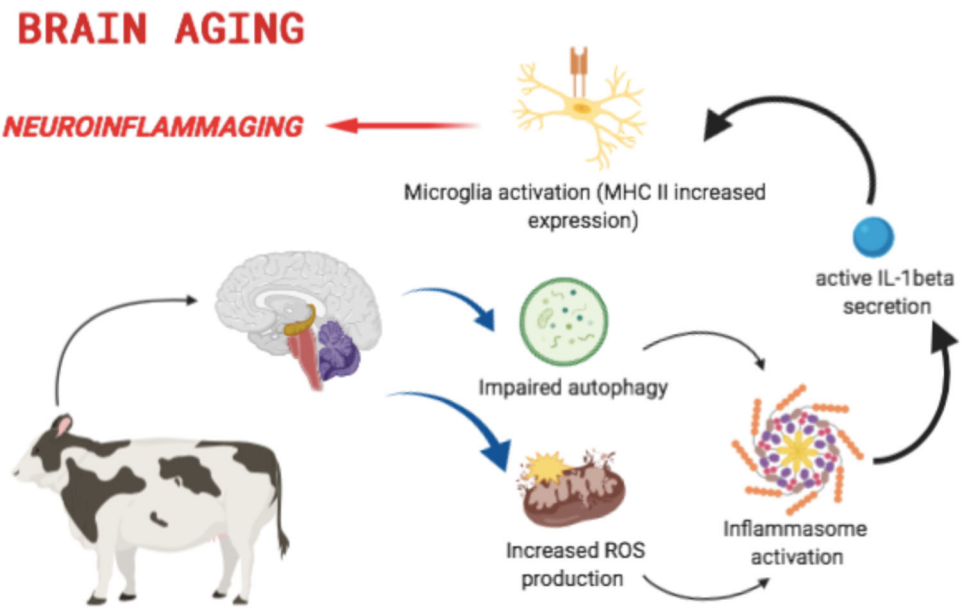

FIGURE 6 Graphical representation of the hypothesized interplay between NLRP3, Autophagy and oxidative stress in neuroinflammation in aged bovine brains

NLRP3 components are induced during the first, priming signal through the toll-like receptor (TLR)-NF-κB pathway (Chang et al., 2015; Zhou, Shi, Wang, Chen, & Zhang, 2016). The subsequent assembly of NLRP3 inflammasome complex occurs during a second signal that can be triggered by several stimuli, both exogenous (including infection, tissue damage, and metabolic dysregulation) and endogenous (including the production of ROS, mitochondrial dysfunction, and accumulation of molecules such as extracellular ATP, hyaluronan, Aβ fibrils, and uric acid crystals; Chang et al., 2015; Walsh et al., 2014; Zhou et al., 2016).

Moreover, the role of autophagy in inflammasome activation in the aging of CNS and in CNS diseases has achieved substantial recognition and new research are continually emerging (Walsh et al., 2014). Autophagy is an ancient, self-degradative, highly regulated housekeeping mechanism which controls the nonspecific degradation of cytoplasmic macromolecules and organelles via the lysosomal system. Autophagy preserves the cellular homeostasis by facilitating the removal of misfolded proteins and dysfunctional organelles (De Biase et al., 2017; Ravikumar et al., 2010). Normally, the autophagic uptake of dysfunctional mitochondria prevents the excessive ROS production and the consequent activation of inflammasomes (Salminen, Kaarniranta, & Kauppinen, 2012). However, during aging, the autophagic capacity declines and increased ROS production may activate inflammasomes which provoke a low-grade inflammation inhibiting autophagy and accelerating the aging process (Salminen et al., 2012). Based on the hypothesis that the age-related impairment in cellular housekeeping mechanisms and the increased oxidative stress can cause neuroinflammation, we investigated the potential interactions between autophagy impairment, increased ROS production, and the subsequent activation of the inflammatory danger sensor NLRP3. For this study, Beclin1 was used as a marker for autophagy and the results were similar to those previously described (De Biase et al., 2017). The overall expression of Beclin1 was reduced in aged animals compared with younger animals. Occasionally, neurons of mature and aged animals showed unexpected coexpression of Beclin1 and NLRP3 inflammasome. This finding apparently goes against the grain, but several authors have recently suggested that the activation of inflammasomes leads to an induction of autophagy that works to limit inflammasome activity by physical engulfment (Martins et al., 2015). Although it is well established that autophagy downregulates the inflammasome activity, their relationship may be more multifaceted (Salminen et al., 2012). In their pivotal work, Martins and colleagues realistically suggested that the role of autophagy in regulating the inflammasome may depend on time and context: In the absence of a danger signal, autophagy may act to remove pro-IL-1β and inflammasome components from the cell, thus maintaining cellular homeostasis (Martins et al., 2015).

SOD1 is an enzyme normally used by cells to protect themselves against oxygen-free radicals. Here, we describe a neuronal overexpression of SOD1 in aged and adult animals compared with younger. Moreover, double-color immunofluorescence indicated a likely positive association between NLRP3 and SOD1 expression. We suggest that the age-related overexpression of SOD1 may imply an excessive dismutase activity due to an overproduction of ROS. The increased production of ROS may exceed the antioxidant capacity of SOD1 resulting in deleterious peroxidative reactions, production of highly toxic hydroxyl radicals, and ultimately oxidative stress-related injury (Jaarsma et al., 2000) and neuroinflammation (Singhal et al., 2014).

NLRP3 is also known for its critical role in the processing of pro-IL-1β and pro-IL-18 in their biologically active form (Jha et al., 2010). These cytokines are key mediators and modulators of inflammatory diseases and are important players in several pathologic mechanisms involved in infection and cancer (Zhu & Kanneganti, 2017). Several authors demonstrated that IL-18 is constitutively expressed in intestinal epithelial cells and in keratinocytes under steady state of healthy humans and furthermore in the blood

monocytes and in murine macrophages, dendritic cells, endothelial cells, intestinal epithelial cells (Dinarello, 2007; Dinarello & Giamila, 2003; Netea et al., 2006; Park, Cheon, & Cho, 2007). Soon after its discovery and cloning, authors were still uncertain about the expression of IL-18 in the CNS. Nowadays, It is widely accepted that IL-18 could be synthesized centrally and that its receptor subunits can be broadly expressed in neurons. However, its role in brain health and disease is still elusive (Alboni, Cervia, Sugama, & Conti, 2010).

In contrast to IL-18, IL-1β is not constitutively expressed under homeostasis and its expression is induced in blood mononuclear cells, macrophages, and dendritic cells during stimulation with TLRs ligands and other cytokines (Zhu & Kanneganti, 2017). With this study, we tested the initial hypothesis that the release and secretion of IL-18 and IL-1β followed the age-dependent NLRP3 inflammasome increased activation. As expected, by molecular analysis we found an age-related increased secretion of IL-1β in the brain of adult and aged animals compared with young. IL-1β, a member of the IL-1 cytokine family, is considered to be a major proinflammatory cytokine in the brain and it is suggested to play a key role in the progress of several neurodegenerative diseases in humans, such as Alzheimer's disease (AD; Wang, Tan, Yu, & Tan, 2015). Furthermore, chronic neuroinflammation sustained by IL-1β has also been implicated in the stereotypical morphology of β-amyloid plaques and fibrillary tangles found in AD (Wang et al., 2015). However, it is yet to be elucidated whether chronic IL-1β elevation participates in or is a direct consequence of brain "normal" or pathologic aging (Wang et al., 2015).

IL-18 is also a source of inflammation and its upregulation can have harmful effect on brain: It drives the local production and secretion of the biologically active form of IL-1β and it also increases IL-18 gene expression which may play an important role in the pathogenesis of AD in humans as well as in other neurodegenerative and neuropsychiatric disorders, (Alboni et al., 2010; Sutinen, Pirttilä, Anderson, Salminen, & Ojala, 2012). Unlike IL-1β, our results showed that IL-18 expression levels were significantly elevated only in the brains of adult animals whereas aged and young groups did not show any upregulation. The discrepancy in cytokine expression is an observation in line with other authors reporting that IL-18 and IL-1β are differentially regulated and that their secretion follows a distinct licensing in response to NLRP3 inflammasome activation (Schmidt & Lenz, 2012; Zhu & Kanneganti, 2017). Studies on AD in humans have shown that IL-18 levels change according to the severity of the disease: Motta, Imbesi, Di Rosa, Stivala, and Malaguarnera (2007) interestingly described higher IL-18 expression in AD-mild patients, lower IL-18 expression in AD-moderate patients and no significant difference between AD-severe patients and nondemented age-matched subjects. The authors suggest that the increase of cytokine levels in mild and moderate-AD patients reflects the functional systemic responsiveness of the immune cells to the inflammatory stimuli elicited by Aβ (Motta et al., 2007). Even though our previous studies in aged bovine brains did not find any morphological lesion equivalent to AD, we described an increased presence of amyloid precursor protein (APP) in the brains of the elderly (De Biase et al., 2017). The diminished production of IL-18 in aged brains may be associated with an impaired phagocytic activity (Motta et al., 2007) also suggesting a gradual decline of immune responsiveness. Future studies may establish whether the high levels of IL-18 found in adult animals exert protective or detrimental effects in aging and if inflammation might promote APP deposition or vice versa.

Finally, microglial activation was evaluated by performing immunohistochemistry for MHC II. Our results revealed a distinct difference between the three studied groups indicating a significant increased microglial activation in the hippocampus of mature and aged cows even though they did not show any apparent neurological disorder. Microglia are often referred to as the resident innate immune cells of the CNS because, similar to macrophages, they can provide the first and main form of active immune defense in the brain (Norden & Godbout, 2013). Microglia respond to and propagate inflammatory signals initiated at the periphery coordinating the immune response to infection (Hanisch & Kettenmann, 2007). When activated, microglia are capable of acquiring diverse and complex phenotypes which permits them to participate in the cytotoxic response, immune regulation, and injury resolution (Liu & Quan, 2018; Ward et al., 2015). With normal aging, microglia develop an inflammatory phenotype revealing increased release of proinflammatory cytokines and an increased expression of MHC II and CD86, scavenger receptors (CD68), pattern-associated recognition receptors, (e.g., TLRs) and integrins (CD11b and CD11c; Norden & Godbout, 2013). Interestingly, in the aged brain microglial activation is amplified and prolonged, which may be related to impairments in several key regulatory systems (Norden & Godbout, 2013). It has yet to be determined if the persistent activation of microglia is a normal aging event or it is due to a periodic stimulation of the immune system. However, several authors suggest that an increase in activation level over time may severely alter the microglial responses to challenges, thus making the brain more susceptible to neurological damage and actively participating in the initiation and progression of neurodegenerative diseases (Fumagalli, Lecca, & Abbracchio, 2011).

Our study introduces intriguing observations, but it has opened a plethora of research opportunities to investigate inflammasome and age-related changes in the bovine brain.

5 | CONCLUSIONS

With this study, we explored "neuro-inflammaging," one of the most recognized effects of brain aging. Our results support the hypothesis that the age-related dysregulation of autophagy and increased oxidative stress may act as stimuli for the maturation and activation of NLRP3 inflammasome. The activation of NLRP3 inflammasome causes the secretion of inflammatory cytokines such as IL-1β with the subsequent priming of the innate immune response and microglial activation, thus contributing to a proinflammatory environment. In our opinion, this study reveals results that are consistent with studies previously performed in other animal models as well as humans.

However, we would like to highlight that bovine may potentially be a pivotal animal model for brain aging studies.

ACKNOWLEDGMENT

The authors would like to thank Dr. Arianna Ilsami for her valuable technical support.

AUTHOR CONTRIBUTIONS

D. D. B. and O. P. conceived and designed the experiments. E. G. performed ante-mortem physical examination and post-mortem inspection of the animals. D. D. B., G. P., and F. P. performed the histological experiment and wrote the paper. I. C. and C. P. performed PCR and western blot analysis. D. D. B., G. P., and F. P. analyzed the data. G. M. R., F. O., and S. P. contributed to the critical reading of the paper. All authors read and gave their approval for the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

ORCID

Davide De Biase http://orcid.org/0000-0002-8118-9807

REFERENCES

Alboni, S., Cervia, D., Sugama, S., & Conti, B. (2010). Interleukin 18 in the CNS. *Journal of Neuroinflammation*, *7*, 9. https://doi.org/10.1186/1742-2094-7-9

Batatinha, H. A. P., Diniz, T. A., de Souza Teixeira, A. A., Krüger, K., & Rosa-Neto, J. C. (2019). Regulation of autophagy as a therapy for immunosenescence-driven cancer and neurodegenerative diseases: The role of exercise. *Journal of Cellular Physiology*, *234*, 14883–14895.

Bauernfeind, F., Ablasser, A., Bartok, E., Kim, S., Schmid-Burgk, J., Cavlar, T., & Hornung, V. (2011). Inflammasomes: Current understanding and open questions. *Cellular and Molecular Life Sciences*, *68*(5), 765–783.

De Biase, D., Costagliola, A., Del Piero, F., Di Palo, R., Coronati, D., Galiero, G., … Paciello, O. (2018). Coxiella burnetii in infertile dairy cattle with chronic endometritis. *Veterinary Pathology*, *55*(4), 539–542.

De Biase, D., Costagliola, A., Pagano, T. B., Piegari, G., Wojcik, S., Dziewiątkowski, J., … Paciello, O. (2017). Amyloid precursor protein, lipofuscin accumulation and expression of autophagy markers in aged bovine brain. *BMC Veterinary Research*, *13*(1), 102.

De Biase, D., & Paciello, O. (2015). Essential and current methods for a practical approach to comparative neuropathology. *Folia Morphologica*, *74*(2), 137–149. https://doi.org/10.5603/FM.2015.0024

Directive, E. C. (2009). Council Regulation No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. *Official Journal of the European Union L*, *303*, 1–30.

Chang, Y. P., Ka, S. M., Hsu, W. H., Chen, A., Chao, L. K., Lin, C. C., … Hua, K. F. (2015). Resveratrol inhibits NLRP3 inflammasome activation by preserving mitochondrial integrity and augmenting autophagy. *Journal of Cellular Physiology*, *230*(7), 1567–1579.

Cimmino, I., D'Esposito, V., Liguoro, D., Liguoro, P., Ambrosio, M. R., Cabaro, S., … Valentino, R. (2019). Low-dose Bisphenol-A regulates inflammatory cytokines through GPR30 in mammary adipose cells. *Journal of Molecular Endocrinology*, *63*, 273–283.

Compan, V., Baroja-Mazo, A., López-Castejón, G., Gomez, A. I., Martínez, C. M., Angosto, D., … Pelegrín, P. (2012). Cell volume regulation modulates NLRP3 inflammasome activation. *Immunity*, *37*, 487–500.

Deleidi, M., Jäggle, M., & Rubino, G. (2015). Immune aging, dysmetabolism, and inflammation in neurological diseases. *Frontiers in Neuroscience*, *9*, 172.

Dinarello, C. A. (2007). Interleukin-18 and the pathogenesis of inflammatory diseases. *Seminars in Nephrology*, *27*, 98–114.

Dinarello, C. A., & Fantuzzi, G. (2003). Interleukin-18 and host defense against infection. *The Journal of Infectious Diseases*, *187*(Suppl. 2), S370–S384.

Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., … Cevenini, E. (2007). Inflammaging and anti-inflammaging: A systemic perspective on aging and longevity emerged from studies in humans. *Mechanisms of Ageing and Development*, *128*(1), 92–105.

Fumagalli, M., Lecca, D., & Abbracchio, M. P. (2011). Role of purinergic signalling in neuro-immune cells and adult neural progenitors. *Frontiers in Bioscience*, *16*, 2326–2341.

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., & Gage, F. H. (2010). Mechanisms underlying inflammation in neurodegeneration. *Cell*, *140*(6), 918–934.

Hanisch, U. K., & Kettenmann, H. (2007). Microglia: Active sensor and versatile effector cells in the normal and pathologic brain. *Nature Neuroscience*, *10*(11), 1387–1394.

Harris, J., Lang, T., Thomas, J. P., Sukkar, M. B., Nabar, N. R., & Kehrl, J. H. (2017). Autophagy and inflammasomes. *Molecular Immunology*, *86*, 10–15.

Jaarsma, D., Haasdijk, E. D., Grashorn, J. A. C., Hawkins, R., van Duijn, W., Verspaget, H. W., … Holstege, J. C. (2000). Human Cu/Zn superoxide dismutase (SOD1) overexpression in mice causes mitochondrial vacuolization, axonal degeneration, and premature motoneuron death and accelerates motoneuron disease in mice expressing a familial amyotrophic lateral sclerosis mutant SOD1. *Neurobiology of Disease*, *7*(6), 623–643.

Jha, S., Srivastava, S. Y., Brickey, W. J., Iocca, H., Toews, A., Morrison, J. P., … Ting, J. P. Y. (2010). The inflammasome sensor, NLRP3, regulates CNS inflammation and demyelination via caspase-1 and interleukin-18. *Journal of Neuroscience*, *30*(47), 15811–15820.

Latz, E., Xiao, T. S., & Stutz, A. (2013). Activation and regulation of the inflammasomes. *Nature Reviews Immunology*, *13*(6), 397–411.

Liu, X., & Quan, N. (2018). Microglia and CNS interleukin-1: Beyond immunological concepts. *Frontiers in Neurology*, *9*, 8.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The hallmarks of aging. *Cell*, *153*(6), 1194–1217.

Luo, X. G., Ding, J. Q., & Chen, S. D. (2010). Microglia in the aging brain: Relevance to neurodegeneration. *Molecular Neurodegeneration*, *5*(1), 12.

Martinon, F., Burns, K., & Tschopp, J. (2002). The inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of pro-IL-β. *Molecular Cell*, *10*(2), 417–426.

Martins, J. D., Liberal, J., Silva, A., Ferreira, I., Neves, B. M., & Cruz, M. T. (2015). Autophagy and inflammasome interplay. *DNA and Cell Biology*, *34*(4), 274–281.

Montecino-Rodriguez, E., Berent-Maoz, B., & Dorshkind, K. (2013). Causes, consequences, and reversal of immune system aging. *The Journal of Clinical Investigation*, *123*(3), 958–965.

Morales, I., Guzmán-Martínez, L., Cerda-Troncoso, C., Farías, G. A., & Maccioni, R. B. (2014). Neuroinflammation in the pathogenesis of Alzheimer's disease. A rational framework for the search of novel therapeutic approaches. *Frontiers in Cellular Neuroscience*, *8*, 112.

Mortezaee, K., Khanlarkhani, N., Beyer, C., & Zendedel, A. (2018). Inflammasome: Its role in traumatic brain and spinal cord injury. *Journal of Cellular Physiology*, *233*(7), 5160–5169.

Motta, M., Imbesi, R., Di Rosa, M., Stivala, F., & Malaguarnera, L. (2007). Altered plasma cytokine levels in Alzheimer's disease: Correlation with the disease progression. *Immunology Letters*, *114*(1), 46–51.

Netea, M. G., Joosten, L. A., Lewis, E., Jensen, D. R., Voshol, P. J., Kullberg, B. J., … Meer van der, J. W. (2006). Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance. *Nature Medicine*, *12*(6), 650–656.

Norden, D. M., & Godbout, J. P. (2013). Microglia of the aged brain: Primed to be activated and resistant to regulation. *Neuropathology and Applied Neurobiology*, *39*(1), 19–34.

Pagano, T. B., Wojcik, S., Costagliola, A., De Biase, D., Iovino, S., Iovane, V., … Paciello, O. (2015). Age related skeletal muscle atrophy and upregulation of autophagy in dogs. *The Veterinary Journal*, *206*(1), 54–60.

Park, S., Cheon, S., & Cho, D. (2007). The dual effects of interleukin-18 in tumor progression. *Cellular Molecular Immunology*, *4*(5), 329–335.

Pascarella, A., Terracciano, C., Farina, O., Lombardi, L., Esposito, T., Napolitano, F., … Bernardini, S. (2018). Vacuolated PAS-positive lymphocytes as a hallmark of Pompe disease and other myopathies related to impaired autophagy. *Journal of Cellular Physiology*, *233*(8), 5829–5837.

Ramos, H. J., Lanteri, M. C., Blahnik, G., Negash, A., Suthar, M. S., Brassil, M. M., … Gale, M., Jr (2012). IL-1β signaling promotes CNS-intrinsic immune control of West Nile virus infection. *PLOS Pathogens*, *8*(11), e1003039.

Ratsimandresey, R. A., Dorfleutner, A., & Stehlik, C. (2013). An update on PYRIN domain-containing pattern recognition receptors: From immunity to pathology. *Frontiers in Immunology*, *4*, 440.

Ravikumar, B., Sarkar, S., Davies, J. E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z. W., … Rubinsztein, D. C. (2010). Regulation of mammalian autophagy in physiology and pathophysiology. *Physiological Reviews*, *90*(4), 1383–1435.

Salminen, A., Kaarniranta, K., & Kauppinen, A. (2012). Inflammaging: Disturbed interplay between autophagy and inflammasomes. *Aging*, *4*(3), 166–175.

Schmidt, R. L., & Lenz, L. L. (2012). Distinct licensing of IL-18 and IL-1β secretion in response to NLRP3 inflammasome activation. *PLOS One*, *7*(9), e45186.

Sheffield, L. G., & Berman, N. E. (1998). Microglial expression of MHC class II increases in normal aging of nonhuman primates. *Neurobiology of Aging*, *19*(1), 47–55.

Singhal, G., Jaehne, E. J., Corrigan, F., Toben, C., & Baune, B. T. (2014). Inflammasomes in neuroinflammation and changes in brain function: A focused review. *Frontiers in Neuroscience*, *8*, 315.

Sutinen, E. M., Pirttilä, T., Anderson, G., Salminen, A., & Ojala, J. O. (2012). Pro-inflammatory interleukin-18 increases Alzheimer's disease-associated amyloid-β production in human neuron-like cells. *Journal of Neuroinflammation*, *9*(1), 199.

von Herrmann, K. M., Salas, L. A., Martinez, E. M., Young, A. L., Howard, J. M., Feldman, M. S., Christensen, B. C., & Havrda, M. C. (2018). NLRP3 expression in mesencephalic neurons and characterization of a rare NLRP3 polymorphism associated with decreased risk of Parkinson's disease. *NPJ Parkinson's disease*, *4*(1), 24.

Walsh, J. G., Muruve, D. A., & Power, C. (2014). Inflammasomes in the CNS. *Nature Reviews Neuroscience*, *15*(2), 84–97.

Wang, W. Y., Tan, M. S., Yu, J. T., & Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Annals of Translational Medicine*, *3*(10), 136.

Ward, R. J., Dexter, D. T., & Crichton, R. R. (2015). Ageing, neuroinflammation and neurodegeneration. *Frontiers in Bioscience*, *7*, 189–204.

Wen, H., Miao, E. A., & Ting, J. P. (2013). Mechanisms of NOD-like receptor-associated inflammasome activation. *Immunity*, *39*(3), 432–441.

Yuk, J.-M., & Jo, E.-K. (2013). Crosstalk between autophagy and inflammasomes. *Molecules and Cells*, *36*(5), 393–399.

Zhong, Z., Sanchez-Lopez, E., & Karin, M. (2016). Autophagy, NLRP3 inflammasome and auto-inflammatory/immune diseases. *Clinical and Experimental Rheumatology*, *34*(4), 12–16.

Zhou, K., Shi, L., Wang, Y., Chen, S., & Zhang, J. (2016). Recent advances of the NLRP3 inflammasome in central nervous system disorders. *Journal of Immunology Research*, *2016*, 9238290.

Zhu, Q., & Kanneganti, T. D. (2017). Cutting edge: Distinct regulatory mechanisms control proinflammatory cytokines IL-18 and IL-1β. *The Journal of Immunology*, *198*(11), 4210–4215.

How to cite this article: De Biase D, Piegari G, Prisco F, et al. Autophagy and NLRP3 inflammasome crosstalk in neuroinflammation in aged bovine brains. *J Cell Physiol*. 2020;1–10. https://doi.org/10.1002/jcp.29426