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Isabel María Guijarro Torvisco

# Valoración de la respuesta inflamatoria en el scrapie como prototipo de enfermedad priónica

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Tesis Doctoral

# VALORACIÓN DE LA RESPUESTA INFLAMATORIA EN EL SCRAPIE COMO PROTOTIPO DE ENFERMEDAD PRIÓNICA


Autor

Isabel María Guijarro Torvisco

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Monzón Garcés, Marta

**UNIVERSIDAD DE ZARAGOZA**
**Escuela de Doctorado**

Programa de Doctorado en Medicina y Sanidad Animal


2020

Universidad Zaragoza
1542

# Tesis Doctoral

## Valoración de la respuesta inflamatoria en el scrapie como prototipo de enfermedad priónica


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Facultad de Veterinaria/ Departamento de Patología Animal


2020

Centro de Encefalopatías y Enfermedades Transmisibles Emergentes

Departamento de Patología Animal

Facultad de Veterinaria

# TESIS DOCTORAL:

# VALORACIÓN DE LA RESPUESTA INFLAMATORIA EN EL SCRAPIE COMO PROTOTIPO DE ENFERMEDAD PRIÓNICA

Memoria de tesis presentada por la graduada

**Isabel María Guijarro Torvisco**

para optar al grado de Doctora por la Universidad de Zaragoza

Directores:

Dra. Marta Monzón Garcés

Dr. Juan José Badiola Díez

Zaragoza, junio de 2020

Este trabajo ha sido posible gracias a una ayuda para la formación del profesorado universitario (FPU, convovatoria 2015) del Ministerio de Educación, Cultura y Deporte (**FPU 15/03524**), y gracias a la Universidad de Zaragoza, que ha aportado las instalaciones y a la cual pertenecen los Directores del trabajo. Asimismo, este trabajo ha sido posible gracias al apoyo de los siguientes programas y sus proyectos:

– Programa de Cooperación Transfronteriza España, Francia, Andorra con contribución del Fondo Europeo de Desarrollo Regional (POCTEFA-FEDER): Proyecto "Red de Investigación Transfronteriza en Enfermedades Priónicas Humanas y Animales", EFA 148/16 (Redprion)

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# MENCIÓN INTERNACIONAL
## Aval del director/es

En relación con la solicitud de Mención Internacional en el título de doctor por D/Dª Isabel María Guijarro Torvisco, del programa de doctorado en Medicina y Sanidad Animal, los abajo firmantes, directores de su tesis doctoral, hacen constar que:

Isabel María Guijarro Torvisco, bajo nuestra dirección, presenta el trabajo correspondiente a su Tesis Doctoral titulada **"VALORACIÓN DE LA RESPUESTA INFLAMATORIA EN EL SCRAPIE COMO PROTOTIPO DE ENFERMEDAD PRIÓNICA"** para optar al Grado de Doctora por la Universidad de Zaragoza con "Mención Internacional". Cumpliendo con la legislación vigente, este trabajo incluye dos informes de los siguientes expertos doctores pertenecientes a instituciones extranjeras:

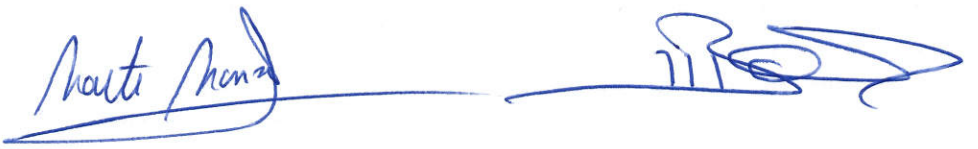
- Dra. Alicia Otero, Postdoctoral fellow, Department of Biological Sciences and Centre for Prions and Protein Folding Diseases, University of Alberta (Edmonton, Alberta, Canadá). Doctora por la Universidad de Zaragoza el 18/09/2018.

- Dra. Helen Caroline Raksa, Lab Manager, Laboratory of Celular Biology, Instituto Carlos Chagas, Fiocruz-Paraná (Curitiba, Paraná, Brasil). Doctora por la Universidad de Zaragoza el 06/06/2018.

Asimismo, avalamos la realización por parte del doctorando de una estancia de investigación en una institución extranjera durante tres meses:

Estancia desde 01 de septiembre hasta el 30 de noviembre de 2019 (90 días) en el INRA ENVT, Interactions-Hôtes-Agents Pathogènes (IHAP), École Nationale Veterinarie de Toulouse (ENVT), Université Fédérale Toulouse Midi-Pyrénées, Toulouse, Francia bajo la supervisión del Dr. Olivier Andrèoletti, en la que la estudiante de doctorado trabajó en el uso de técnicas de diagnóstico molecular y su aplicabilidad a la investigación de las enfermedades priónicas. Por tanto, cumple con los requisitos necesarios para optar al Título de Doctora con Mención Internacional.

Fecha y firma: Zaragoza, a 19 de Junio de 2020



Fdo: Marta Monzón Garcés

Fdo: Juan José Badiola Díez

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SR. PRESIDENTE DE LA COMISIÓN DE DOCTORADO
UNIVERSIDAD DE ZARAGOZA

# INFORME VALORACIÓN TESIS DOCTORAL / PhD THESIS ASSESSMENT REPORT

Doctorando-a /Doctoral Student: Isabel Maria Guijarro Torvisco

Título de la Tesis / Title of the Thesis: VALORACIÓN DE LA RESPUESTA INFLAMATORIA EN EL SCRAPIE COMO PROTOTIPO DE ENFERMEDAD PRIÓNICA

D./Dª.Helen Caroline Raksa……..

☐ Como …… del Tribunal que ha juzgado la tesis doctoral / As …….. of the Doctoral Committee that has evaluated the PhD thesis

■ Como evaluador de la tesis con mención de doctorado internacional o europea / As external examiner of the PhD thesis (International or European Mention PhD

emite el informe que sigue / issues the following report.

.

En Curitiba , a 19 de junio de 2020.

Firma/Sign.: ……...

Informe de Valoración para la Comisión de Doctorado / Evaluation Report.

Comente la originalidad del trabajo presentado, la relevancia del mismo dentro del dominio al que pertenece la tesis, la metodología y la calidad de la memoria presentada. Incluya, en su caso, los comentarios que desee hacer llegar al doctorando (adjunte las hojas que sean necesarias).
Discuss the originality and relevance of the work as well as the quality of the dissertation. You may include any comments that you want to send to the PhD student (please, attach as many pages as necessary).

To Whom It May Concern:

The scientific work presented in this thesis is highly relevant to the field of Scrapie research and makes important contributions to the knowledge the different aspects of the immune response that occurs in different stages of the disease.

The review of the bibliography are ample (48 pages) and reveal are clear understanding. The addressed topics are well chosen and support the rest of chapters as well as the overall conclusions of the thesis.

The Thesis overall objective is approach to the neuroinflammatory process that occurs in the neurodegenerative progress of scrapie and analyse the effect of an anti-inflammatory therapy on clinical and neuropathological parameters. For this, the Thesis contains four high quality studies, with clear objectives, methodology and results.

In the first study, the PhD student evaluetad the staining intensity of diferents cytokines by immunohistochemistry (IHC) in Purkinje cells of cerebellum of sheep affected by natural scrapie at different clinical stages in order to evaluate whether the intensity of their immunostaining vary throughout disease progress. The results of this preliminary study confirms that a complex network of cytokines is involved in the pathogenesis of natural scrapie, thus suggesting a relevant immunological component in prion diseases.

The second study to assessed the effect of the dexamethasone on the spread of scrapie in a natural sheep model, paying special attention to the differential expression of astroglial and microglial markers as main components of the host immune response in the brain. The study results demonstrated how the interaction between glial populations fails to compensate for brain damage in natural conditions and evidence that modulation of neuroinflammation by antiinflammatory drugs may become a research focus as a potential therapeutic target for prion diseases.

The third study assessed the effect of an anti-inflammatory treatment with dexamethasone on different cytokines released by neuroglial cells that are potentially related to neuroinflammation in natural scrapie. The study is the fisrt one a assessing in situ

neuroinflammatory activity in relation with chronic anti-inflammatory therapy, gaining relevance because it is based on a natural model. The study suggested an impaired communication between microglia and astroglia, which is mediated by cytokines, mainly IL-1.

In the fourth study, the author avaliated the effects of dexametasone treatment, in natural scrapie, at initial phases of disease. The results demonstrated glucocorticoid treatment applied directly influences neuroglial response in preclinical sheep naturally affected by scrapie.

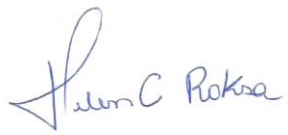
The thesis is appropriately referenced, the experiments have been carried out using the appropriate methodology, the results are well presented and the conclusions are supported by the data provided. The tables and figures are very useful for understanding the studies and the microscopic photographs illustrating the immunohistochemistry are excelente.

I would like to congratulating Isabel María Guijarro Torvisco for her excellent research work and the hard work that implies experiments with animals.

Finally, I have no hesitation to recommend this work to be submitted for examination as a Doctoral Thesis.

June 19, 2020

Yours Sincerely,



Helen Caroline Raksa, PhD
Lab manager, Laboratory of Celular Biology, Instituto Carlos Chagas, Fiocruz – Paraná – Brazil

Ministério da Saúde
FIOCRUZ - PARANÁ
Instituto Carlos Chagas

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1542

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UNIVERSIDAD DE ZARAGOZA

# INFORME VALORACIÓN TESIS DOCTORAL / PhD THESIS ASSESSMENT REPORT

Doctorando-a /Doctoral Student: ISABEL MARÍA GUIJARRO TORVISCO

Título de la Tesis / Title of the Thesis:
VALORACIÓN DE LA RESPUESTA INFLAMATORIA EN EL SCRAPIE COMO PROTOTIPO DE ENFERMEDAD PRIÓNICA

D./Dª ALICIA OTERO GARCÍA

☐ Como …… del Tribunal que ha juzgado la tesis doctoral / As …….. of the Doctoral Committee that has evaluated the PhD thesis

☒ Como evaluador de la tesis con mención de doctorado internacional o europea / As external examiner of the PhD thesis (International or European Mention PhD

emite el informe que sigue / issues the following report.

.

En Edmonton, a 16 de junio de 2020

Firma/Sign.....

Informe de Valoración para la Comisión de Doctorado / Evaluation Report.

Comente la originalidad del trabajo presentado, la relevancia del mismo dentro del dominio al que pertenece la tesis, la metodología y la calidad de la memoria presentada. Incluya, en su caso, los comentarios que desee hacer llegar al doctorando (adjunte las hojas que sean necesarias).
Discuss the originality and relevance of the work as well as the quality of the dissertation. You may include any comments that you want to send to the PhD student (please, attach as many

I have read with great interest the Ph.D. thesis of Ms. Isabel María Guijarro Torvisco entitled "Valoración de la respuesta inflamatoria en el scrapie como prototipo de enfermedad priónica". In the literature review, she deeply revises the general aspects of prion diseases with special attention to scrapie, the disease studied in this thesis. In addition, she thoroughly reviews the role of glial cells and cytokines in neurodegenerative diseases, neuroinflammatory mechanisms and their role in neurodegeneration and the possible application of corticosteroids in the treatment of these processes, given their immunosuppressive properties. This bibliographic review is very complete and includes figures and tables that perfectly summarize the content of the different sections.

This Ph.D. thesis is organized in four studies, one of them already published in the high-impact journal *International Journal of Molecular Sciences*. These four studies perfectly address different mechanisms of the immune response at different stages of scrapie disease. I would like to acknowledge that she studies a possible anti-inflammatory therapy for scrapie, which adds value to this thesis considering that there is no known treatment for prion diseases.

In the first study, she analyzes, by immunohistochemistry, the possible role of the immune response mediated by cytokines in the neurodegeneration that occurs during the pathogenesis of scrapie. It was found that IL-1 and IL-6 are overexpressed in the cerebellum of clinical and terminally ill sheep, whereas IL-1R and IL-2R seem to be downregulated in these animals. She also describes a progressive increase in the expression of IL-10R and TNFαR from the preclinical stage. This study has solid conclusions and suggests a likely role of the immune system in the pathogenesis of scrapie.

The second study entitled "Assessment of host immune response in the progress of natural scrapie after chronic dexamethasone treatment" explores a possible treatment for sheep scrapie. The Ph.D. candidate studies the immunohistochemical expression of glial markers, prion accumulation, neuropathological lesions and the clinical evolution of naturally infected sheep chronically treated with dexamethasone with those of non-treated control sheep. Interestingly, she reports an evident extension of survival times in one case and shows how the interactions between glial populations fail to compensate for brain damage in naturally infected animals. I would like to acknowledge the enormous amount of work that a study of these characteristics implies, with the added difficulty of working with large animals.

In the third study she evaluates the contribution of inflammatory responses mediated by cytokines in the brain of scrapie clinical sheep chronically treated with dexamethasone and in the brain of control animals. She reports an impaired communication between microglia and astroglia, suggesting IL-1 as the possible mediator. This is a very complete study, in which numerous immunohistochemical markers are analyzed, and that emphasizes the complex interactions among several peptides implicated in neuroinflammatory processes.

The fourth study is a continuation of the previous one, evaluating the neuroinflammation-associated markers in sheep in the preclinical stage of scrapie chronically treated with dexamethasone and comparing the obtained results with those observed in control sheep. She reports a great decrease in the intensity of vacuolation and prion deposition in treated animals, accompanied by an outstanding increase in astro and microgliosis. This study proposes the initial stage of the disease as a possible window for the treatment of scrapie.

Overall, this Doctoral Thesis submitted by Ms. Isabel María Guijarro Torvisco shows research work of great quality and importance for the field of neurodegenerative diseases. The results presented in this thesis are the reflection of the hard work and perseverance of the Ph.D candidate. This thesis widely meets the requirements for obtaining a PhD degree, and they have my recommendation to be submitted for examination as a Doctoral Thesis.

Finally, I would like to congratulate Ms. Isabel María Guijarro Torvisco for her tireless effort and for producing a high-quality thesis. I wish her all the best in her career.

June 16, 2020

Yours sincerely,

Alicia Otero García, DVM, PhD
*Postdoctoral fellow*

*Department of Biological Sciences and Centre for Prions and Protein Folding Diseases.*
*204 Brain and Aging Research Building, University of Alberta.*

Edmonton, Alberta T6G 2M8,
e -mail: oterogar@ualberta.ca
Phone: +l 780248-1718

UNIVERSITY OF ALBERTA

Universidad Zaragoza
1542

# CERTIFICADO DE ESTANCIA DE INVESTIGACIÓN

El coordinador del programa de doctorado de la Universidad de Zaragoza en Medicina y Sanidad Animal

CERTIFICA que

D/D.ª Isabel María Guijarro Torvisco, procedente de la Universidad de Zaragoza ha realizado desde el 01-sep-2019 *(fecha de inicio)* hasta el 30-nov-2019 *(fecha final)*

una estancia de investigación enmarcada en el desarrollo de su tesis doctoral en el departamento/instituto/centro de UMR IHAP (Ecole Nationale Veterinarie de T(identifíquese lo que proceda).

Y para que así conste, firmo la presente

en Zaragoza, a de de 20

| El coordinador del programa de doctorado | Visto bueno del responsable/tutor Durante la estancia |
|---|---|
| Fdo.: | Fdo.: Olivier Andréoletti |

Escuela de Doctorado
Universidad Zaragoza

# ESTANCIA INVESTIGACIÓN
## Informe de la estancia

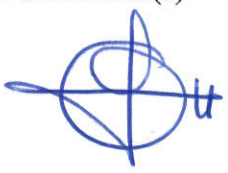
| DOCTORANDO | |
|---|---|
| Apellidos, nombre: Guijarro Torvisco, Isabel María | |
| E-mail: isabelmariagt91@gmail.com | NIP: 732227 |
| Programa de doctorado: Medicina y Sanidad Animal | |

| INFORME | |
|---|---|
| Centro de acogida: INRA ENVT, Interactions-Hôtes-Agents Pathogènes (IHAP), École Nationale Veterinarie de Toulouse (ENVT), Université Fédérale Toulouse Midi-Pyrénées. | |
| Responsable de la estancia: Olivier Andrèoletti | |
| Fecha de inicio: 01-09-2019 | Fecha de finalización: 30-11-2019 |

Durante mi estancia de tres meses en Toulouse me especialicé en el uso de técnicas de diagnóstico molecular (PMCA, Dot Blot y Western Blot) y su aplicabilidad a la investigación de las enfermedades priónicas, llevándolas a cabo sobre muestras de algunos de los animales incluidos en esta tesis doctoral para diagnosticar presencia de priones en tejidos que mediante las técnicas inmunohistoquímicas rutinarias eran difíciles de demostrar.

Zaragoza, a 20 de Junio de 2020.

El/la doctorando(a)



Fdo.: Isabel María Guijarro Torvisco

A mis padres,

A mis hermanos,

A Alejandro

*“Science is organized knowledge. Wisdom is organized life.”*

Immanuel Kant

# AGRADECIMIENTOS

Estas palabras van dedicadas a todas aquellas personas que de una u otra forma han aportado su granito de arena a esta tesis, ya sea en la parte científica o en la personal. Debo resaltar que esto es el fruto de un trabajo en equipo, no individual, ya que todas las aportaciones que he conseguido de todos vosotros han contribuido a tener por fin este manuscrito en las manos después de casi cinco años de aventura doctoral.

Agradezco a mis directores de tesis, la Dra. Marta Monzón Garcés y el Dr. Juan José Badiola Díez:

A Marta, por transmitirme tus conocimientos sobre neuropatología, tu énfasis por la ciencia y demostrarme que todo acaba saliendo, sólo es cuestión de esfuerzo y ganas. Muchas gracias por valorar todo mi tiempo y esfuerzo pero aun así enseñarme a que siempre hay que ser autocrítica.

A Juan, ya que sin ti no hubiera venido a Zaragoza. Me animaste a embarcarme en esta aventura desde el primer momento en que hablamos, abriéndome las puertas de tu laboratorio y queriendo integrarme en tu equipo aun sin conocerme. Simplemente gracias. Espero haber hecho un buen trabajo durante todo este tiempo y que estés orgulloso de haber depositado toda tu confianza en mí.

Al Ministerio de Educación, Cultura y Deporte, por concederme un contrato de investigación predoctoral durante 4 años y además financiarme las estancias breves de Barcelona y Toulouse.

A todo el personal del Centro de Encefalopatías y Enfermedades Transmisibles Emergentes:

A Rosa, Cristina, Eva, Nico, Hicham y Mamen, por ofrecerme vuestra mano tanto en temas de gestión como en Anatomía Patológica siempre que la he necesitado.

A Antonia, por brindarme la oportunidad de impartir docencia en Anatomía Patológica General y enseñarme a tratar con los alumnos de una forma amena y cercana.

A Belén, por tu incalculable ayuda con todo el trabajo de campo y por todos esos viajes en furgoneta; el manejo experimental de los animales y las necropsias no habrían sido posibles sin ti.

A las secretarias, África y Lourdes, toda la parte administrativa cada vez más en auge actualmente habría sido imposible sin vosotras. Gracias también por los ratitos de café en el office.

A los técnicos de laboratorio, Sonia, Sandra y Dani, por la inestimable ayuda con los cortes del microtomo y con cualquier duda que me surgiera en el laboratorio. A Raquel y Cristina, por abrirme vuestro círculo de amigos desde mis primeros meses en Zaragoza e integrarme como una más de vuestra familia.

A mis compañeros que conocí cuando empecé en 2015 y han ido terminando: Carlos, José Luis, Helen, Alicia, Óscar, Moisés y Tomás. De todos aprendí un poco (o mucho) de cómo enfrentar esta aventura. Especial agradecimiento a Helen y Alicia, por haberse leído la tesis y realizado los informes internacionales en tiempo récord y a Tomás, por su incalculable ayuda en temas de diseño y burocracia.

A mis compañeros actuales, a los cuales abandono mientras ellos siguen con su aventura particular: Mirta, Marina y Diego. Porque más que compañeros somos una pequeña familia priónica. Jamás olvidaré las comidas en cafetería o en el office, los congresos, las tardes de cine, los juepinchos, todas las champanadas y patrones, las runizares, las cenas de Navidad, los picoteos de tesis, las videollamadas en confinamiento, los jueves de cónclaves en distintos restaurantes y alguna que otra fiestecilla. Muchísimo ánimo para lo que os queda, intentaré volver para veros evolucionar uno a uno.

Al Dr. Isidre Ferrer y todo su equipo, por tratarme como a una más en Barcelona y hacer que la estancia fuera tan provechosa como motivadora. Especial agradecimiento a Pol y Marga, por todos los cotilleos, comilonas y hacer que esta fuera una experiencia tan sumamente divertida. Además, agradezco a Pol el diseño de la portada y las recetas secretas. A David y Alba por hacerme sentir verdaderamente como en casa.

Al equipo del INRA-ENVT de Toulouse, por acogerme como a una más sin ni siquiera entendernos mutuamente el idioma. En especial a Olivier (por sus bromas, por hacerme sentir

cómoda y ofrecerme su apoyo y ayuda en todo momento), Didier (por recibirme siempre con una sonrisa y con ganas de ayudarme), Claudia (sin ti mi estancia definitivamente no habría sido ni la mitad de divertida y provechosa de lo que fue) y Ana (por los cotilleos y porque aunque no hacías ruido, sé que estabas).

A mis compis del departamento: Javi, Ricardo, Sofía, Raúl y Ana, por transmitirme esa pasión por la Anatomía patológica, además de alguna que otra comida o fiestecilla. A Charlie, Paz, Paula, Eloisa y Adelaida por compartir momentos de comidas, tesis y congresos.

A mis compañeras de piso y a Julia, porque aún sin vivir juntas, has sido una más de nosotras. Esta aventura maña no habría sido tan movidita sin vuestra presencia. En especial a Cantia, Pili y Mónica, por aguantarme en mis días malos y también buenos, con muchas fiestas, juepinchos, viajes, cines o simplemente domingos de sofá. Por hacer que nos sintiéramos "en casa" aun estando todas tan lejos de nuestras familias. También agradezco a las otras compis que, aunque pasaron poco tiempo en casa, dejaron huella y buenos recuerdos (Soumaya, Izabella y Mariela).

A mis amigas (Natalia, Belén, Noelia, Julia, Lourdes, Fabiana, Inés, Jara, Sara, Isa, Paula, Maris, Maria José) y amigos (Fran, Chinchi, Matías, Manu, Diego, Jay), ya que aun en la distancia hemos seguido en contacto y cada reencuentro ha sido como si el tiempo no pasara entre nosotros, aportándome un poquito más de energía para seguir con esta batalla.

A las ovejas, indudablemente sin ellas no hubiera sido posible esta tesis doctoral. Todo lo que sufrí y sufristeis por fin ha merecido la pena.

A Alejandro, por haberte cruzado en mi camino cuando menos lo esperaba. Me has animado y trabajado conmigo desde el primer día y especialmente en esta última fase que ha sido la más estresante. Todos nuestros viajes y visitas me han ayudado a estar hoy aquí.

A mi familia: mis tíos, primos, abuelos (siempre en mi recuerdo) y hermanos. A mis padres, gracias infinitas, me animasteis a venir a Zaragoza el primer año y me habéis aguantado aun en la distancia todo este tiempo. Sin vosotros hoy no sería quien soy.

En definitiva, muchísimas gracias a todos por hacerlo posible, yo también espero haberos aportado cosas buenas durante todo este proceso.

Isabel María Guijarro Torvisco.

# LISTA DE ABREVIATURAS

A: Alanina

ACTH: Hormona adenocorticotropa / corticotropina

*ALDH1L1: Aldehyde dehydrogenase 1 family member L1*

*AIF-1: Allograft inflammatory factor-1*

AINE: Antiinflamatorio no esteroideo/ *NSAID: Non-steroidal anti-inflammatory drug*

BHE: Barrera hematoencefálica/ *BBB: Blood-brain barrier*

*Cb: cerebellum*

CDF: Células dendríticas foliculares

*CJD: Creutzfeldt-Jakob disease*

CRH: Hormona liberadora de corticotropina

*DB: Dot Blot*

DEX: Dexametasona/ *Dexamethasone*

DFTL: Demencia frontotemporal lobar

EA: Enfermedad de Alzheimer/ *AD: Alzheimer's disease*

EH: Enfermedad de Hungtington/ *HD: Hungtington's disease*

ELA: Esclerosis lateral amiotrófica/ *ALS: Amyotrophic lateral sclerosis*

EM: Esclerosis múltiple/ *MS: Multiple sclerosis*

EP: Enfermedad de Parkinson/ *PD: Parkinson's disease*

EEB: Encefalopatía espongiforme bovina

eECJ: Enfermedad de Creutzdfeldt-Jacob esporádica

*Fc: Frontal cortex*

GA: Glándula adrenal

GC: Glucocorticoides/ *Glucocorticoids*

gECJ: Enfermedad de Creutzdfeldt-Jacob genética

GFAP: Proteína ácida fibrilar glial/ *Glial fibrillary acidic protein*

*GILZ: Glucocorticoid-induced leucine zipper*

GR: Receptor de glucocorticoides/ *Glucocorticoid receptor*

GSS: Síndrome de Gerstmann-Sträussler-Scheinker

*GUS-β: β-Glucuronidase*

H: Histidina

H-E: Hematoxilina-eosina/ *Haematoxylin eosin*

HPA: Eje hipotálamo-hipófisis-adrenal

*HPRT-1: Hypoxanthine phosphoribosyl transferase-1*

iECJ: Enfermedad de Creutzdfeldt-Jacob iatrogénica

*IBA-1: Ionized Calcium-Binding Adaptor Molecule-1*

IHQ: Inmunohistoquímica/ *IHC: Immunohistochemistry*

IL-1α: Interleuquina 1 alfa/ *Interleukin 1 alpha*

IL-1β: Interleuquina 1 beta/ *Interleukin 1 beta*

IL-1R: Receptor de interleuquina 1/ *Interleukin 1 receptor*

IL-2: Interleuquina 2/ *Interleukin 2*

IL-2R: Receptor de interleuquina 2/ *Interleukin 2 receptor*

IL-6: Interleuquina 6/ *Interleukin 6*

IL-6R: Receptor de interleuquina 6/ *Interleukin 6 receptor*

IFNγ: Interferón gamma/ *Interferon gamma*

IFNγR: Receptor de interferón gamma/ *Interferon gamma receptor*

IL-10: Interleuquina 10

IL-10R: Receptor de interleuquina 10

K: Lisina

LPS: Lipopolisacárido

MHC I: Complejo mayor de histocompatibilidad tipo uno

MHC II: Complejo mayor de histocompatibilidad tipo dos

*MO: Medulla oblongata*

Nor98: Scrapie atípico

*O: Obex*

OIE: Organización Mundial de Sanidad Animal

*PMCA: Protein misfolding cyclic amplification*

PP: Placas de Peyer

PRNP: Gen de la proteína prion

$PrP^{c}$: Proteína prión celular

$PrP^{sc}$: Proteína prión patológica

Q: Glutamina

R: Arginina

RAMALT: *Rectoanal mucosa-associated lymphoid tissue*

RT-qPCR: Reacción en cadena de la polimerasa a tiempo real / *Real time quantitative polimerase chain reaction*

SLR: Sistema linforreticular

SNC: Sistema nervioso central/ *CNS: Central nervous system*

*SSF: Saline solution*

TLR: Receptor tipo toll

TNFα: Factor de necrosis tumoral alfa/ *Tumor necrosis factor alpha*

TNFαR: Receptor del factor de necrosis tumoral / *Tumor necrosis factor alpha receptor*

*TSE: Transmissible spongiform encephalopaties*

V: Valina

vECJ: Enfermedad de Creutzdfeldt-Jacob variante

WB: *Western blot*

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**ESTUDIO Nº 1**



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**ESTUDIO Nº 4**

# I. RESUMEN

# I. RESUMEN

Las enfermedades priónicas son un conjunto de enfermedades neurodegenerativas con largos periodos de incubación y para las cuales actualmente no existe tratamiento.

Tradicionalmente, se ha considerado que no existía una respuesta inmunológica por parte del individuo afectado frente al agente causal de estas enfermedades debido a que no existe infiltración linfocitaria. Sin embargo, en los últimos años se ha demostrado que en todas las enfermedades neurodegenerativas existe un proceso de neuroinflamación que puede conducir a la neurodegeneración. La hipótesis neuroinflamatoria está basada en el hecho de que un estímulo patológico (el depósito de proteína prion) va a provocar la activación de la neuroglía, fundamentalmente astroglía y microglía, que a su vez van a liberar una serie de mediadores inflamatorios en el encéfalo pudiendo contribuir al progreso neurodegenerativo. Aunque la principal función de las células gliales ha sido tradicionalmente considerada la neuroprotección, aún no está claro cuál es su papel real, ya que también existen evidencias de que podrían ejercer un efecto potenciador de la neurodegeneración, convirtiéndose en neurotóxicas. Por lo tanto, dicha hipótesis pone de manifiesto que las células gliales pueden representar el objetivo de estudios dirigidos a establecer una diana terapéutica potencial que pudiera ralentizar la neurodegeneración.

Con este escenario, en esta tesis doctoral se han llevado a cabo cuatro estudios para abordar diferentes aspectos de la respuesta inmunológica que se produce en distintos estadios de la enfermedad de scrapie, prototipo de estudio de las enfermedades priónicas. El objetivo es realizar una aproximación global al proceso neuroinflamatorio que se produce en el progreso neurodegenerativo de esta enfermedad, además de analizar el efecto de una terapia antiinflamatoria sobre parámetros clínicos y neuropatológicos, definiendo para ello las variaciones morfológicas y de intensidad de las células astro y microgliales, así como de varias citoquinas como marcadores neuroinflamatorios que éstas liberan, para poder comprender mejor su comportamiento durante el proceso neurodegenerativo.

Se ha desarrollado un modelo de terapia crónica con el glucocorticoide sintético dexametasona para evaluar el efecto del fármaco sobre la respuesta neuroglial, confirmándose la delicada interacción existente entre las poblaciones astroglial y microglial. Se ha demostrado que el tratamiento ha sido efectivo en la reducción de la vacuolización y el depósito de proteína prión patológica en la fase temprana (en la que aún existe una respuesta neuroglial intacta), mientras que parece existir una parálisis y/o astenia astroglial en el estadio más

avanzado de scrapie (cuando presentan sintomatología clínica), confirmándose que las terapias antiinflamatorias pueden presentar potencial terapéutico solamente en estados incipientes. Sin embargo, resulta esperanzadora la extensión del periodo de supervivencia observada en una de las ovejas clínicas tratadas, al abrir la posibilidad de que estos fármacos puedan tener eficacia al menos en algunos casos clínicos.

Dentro de los objetivos planteados, también se pretendía evaluar la expresión de varias citoquinas y/o receptores en diferentes áreas encefálicas en el progreso del scrapie. Las observaciones realizadas confirman la existencia de una respuesta inmunológica a nivel local a través de una compleja red de citoquinas, sugiriendo que la neuroglía intenta restablecer la homeostasis del encéfalo en las fases iniciales de la enfermedad, pero surge un fallo funcional en estas poblaciones celulares conforme avanza la neurodegeneración, probablemente de la comunicación entre astroglía y microglía, mediado por distintas citoquinas, de forma especial la IL-1.

En definitiva, el conjunto de los resultados obtenidos en los cuatro estudios incluidos en esta tesis doctoral contribuyen a la elaboración del patrón neuroinflamatorio global que sucede en el progreso del scrapie ovino natural. Además, el modelo de este grupo de enfermedades podría ser extrapolable al proceso neurodegenerativo que sucede en el resto de enfermedades priónicas y prion-*like*, en las que también existe un depósito de proteína aberrante como estímulo patológico.

# II. SUMMARY

# II. SUMMARY

Prion diseases are a group of neurodegenerative diseases with long incubation periods and for which there is currently no available treatment.

Traditionally, it has been considered that there was no immune response by the affected individual against the causal agent of these diseases because no lymphocytic infiltration exists. However, in the latest years it has been shown that neuroinflammation is a common process in all neurodegenerative diseases that probably leads to neurodegeneration. The neuroinflammatory hypothesis is based on the fact that a pathological stimulus (prion protein deposit) produces the activation of neuroglia, mainly astroglia and microglia, which in turn release a series of inflammatory mediators in the brain that could contribute to the neurodegenerative progress. Although neuroprotection has traditionally been the main function of glial cells, it is still unclear what their real role is, as there is also evidence that they could be potentiating the effect of neurodegeneration, becoming neurotoxic. Therefore, this hypothesis shows that glial cells may represent the objective of studies aimed to establish a potential therapeutic target that could slow down neurodegeneration.

With this scenario, four studies have been included in this doctoral thesis in order to address different aspects of the immune response that occurs in different stages of scrapie, the prototype of study of prion diseases. The objective is to draw a global approach to the neuroinflammatory process that occurs in the neurodegenerative progress of this disease, as well as to analyse the effect of an anti-inflammatory therapy on clinical and neuropathological parameters, thus defining morphological and intensity variations of astroglial and microglial cells, as well as various cytokines as neuroinflammatory markers that they release in order to better understand their behaviour during the neurodegenerative process.

A model of chronic anti-inflammatory therapy with the synthetic glucocorticoid dexamethasone has been developed in order to evaluate its effect on the neuroglial response, confirming the delicate interaction between the astroglial and microglial populations. The treatment has been shown to be effective in reducing vacuolation and pathological prion protein deposition in the early phase (when there is still an intact neuroglial response), whereas it seems to have paralysis and / or astroglial asthenia in the most advanced stage of scrapie (when animals present clinical symptoms), confirming that anti-inflammatory therapies may have therapeutic potential only in incipient states. However, the extension of the survival

period observed in one of the treated clinical sheep results encouraging, since it opens up the possibility that these drugs may have potential at least in some clinical cases.

In this thesis, it was also intended to evaluate the expression of several cytokines and / or receptors in different brain areas in the progress of scrapie. The made observations confirm the existence of an immunological response at local level throughout a complex network of cytokines, suggesting that neuroglia tries to re-establish brain homeostasis at the early stages of the disease, but a functional failure comes up in these cell populations as neurodegeneration progresses, probably coming from communication between astroglia and microglia, mediated by different cytokines, especially IL-1.

In short, the set of results obtained in the four studies included in this doctoral thesis contribute to elaborate the global neuroinflammatory profile that occurs in the progress of natural ovine scrapie. In addition, the model of this group of diseases could be extrapolated to the neurodegenerative process of the rest of prion and prion-*like* diseases, in which there is also an aberrant protein deposit as pathological stimulus.

# III. REVISIÓN BIBLIOGRÁFICA

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## 1. ENFERMEDADES PRIÓNICAS. GENERALIDADES.

Las enfermedades priónicas son enfermedades neurodegenerativas crónicas y letales de baja incidencia y distribución universal que afectan tanto a la especie humana como a varias especies animales. En cuanto a la causa de estas enfermedades, la teoría más aceptada sostiene que es una isoforma de la proteína prión celular ($PrP^{c}$), con características específicas asociadas a un cambio conformacional (denominada $PrP^{sc}$), la que provoca todas estas enfermedades (Prusiner, 1982). Los priones son partículas proteináceas desprovistas de ácido nucleico (Prusiner, 1998) que puede agregarse, así como reclutar y convertir la $PrP^{c}$ fisiológica en su isoforma patológica (Aguzzi and Calella, 2009).

Se considera que existe un cambio conformacional de la estructura terciaria de la $PrP^{C}$, rica en α-hélice, a una isoforma aberrante $PrP^{Sc}$, rica en β-lámina, disminuyendo su solubilidad y aumentando su resistencia a las proteasas (Figura 1). Se piensa que esta conversión se produce en la superficie celular o a través de distintas rutas endocíticas celulares (Mabbott, 2017; Fehlinger et al., 2017), requiriendo ambas isoformas para su propagación (Sailer et al., 1994). También se ha propuesto la existencia de productos intermedios como causantes de la toxicidad en relación con este cambio conformacional (Ironside et al., 2014).

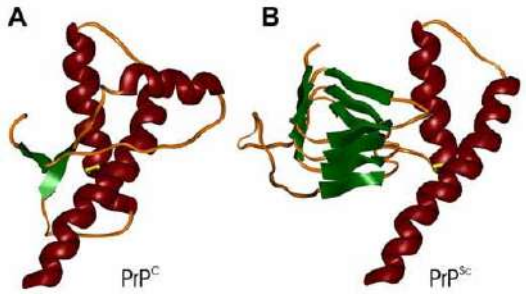


**Figura 1.** Imagen obtenida por análisis de cristalografía electrónica de la estructura de $PrP^{c}$ **(A)** y su isoforma patológica $PrP^{sc}$ **(B)** (Govaerts et al., 2004)

Además de la acumulación de $PrP^{sc}$, este grupo de enfermedades comparten otras características anatomopatológicas: degeneración espongiforme (Prusiner, 1998), pérdida neuronal y gliosis (incluyendo astrocitosis y microgliosis)(DeArmond and Prusiner, 1995; Wadsworth and Collinge, 2011).

Existen estudios recientes que han demostrado que las enfermedades neurodegenerativas humanas, como la enfermedad de Alzheimer (EA), enfermedad de Parkinson (EP), la esclerosis lateral amiotrófica (ELA), la demencia frontotemporal lobar (DFTL) o la enfermedad de Hungtington (EH), comparten precisamente la acumulación de proteínas incorrectamente plegadas o aberrantes tales como β-amiloide, α-sinucleína, TDP-43 o huntingtina (Aguzzi et al., 2008; Fernández-Borges et al., 2013; Prusiner, 2013). Se han evidenciado diversos aspectos a nivel histopatológico y molecular también en común con las enfermedades priónicas (Garcés et al., 2019), por lo que a todos estos desórdenes neurodegenerativos humanos se les ha atribuido el término de patologías prion-*like*. Esto convierte a las enfermedades priónicas en un modelo válido para abordar el proceso patogénico de la neurodegeneración en todas estas proteinopatías.

Hasta ahora se han descrito varias enfermedades priónicas que afectan a un gran número de especies animales domésticas y salvajes (Tabla 1), además del hombre (Tabla 2).

**Tabla 1.** Enfermedades neurodegenerativas causadas por priones en animales domésticos y salvajes (modificada de Prusiner, 2013; Ironside et al., 2017).

| ENFERMEDAD | HOSPEDADOR | AÑO DE LA DESCRIPCIÓN |
|---|---|---|
| **Scrapie** | Oveja, cabra y muflón | 1732 |
| **Scrapie atípico (Nor98)** | Oveja y cabra | 1998 |
| **Encefalopatía espongiforme bovina (EEB)** | Vaca | 1986 |
| **Encefalopatía espongiforme bovina amiloidótica (tipo L)** | Vaca | 2004 |
| **Encefalopatía espongiforme bovina atípica (tipo H)** | Vaca | 2004 |
| **Enfermedad caquectizante del ciervo** | Ciervo, reno y alce | 1967 |
| **Encefalopatía espongiforme felina** | Gato y felinos salvajes (guepardo, puma, tigre, ocelote) | 1990 |
| **Encefalopatía transmisible del visón** | Visón | 1947 |
| **Encefalopatía espongiforme de ungulados exóticos** | Niala, antílope y bisonte | 1986 |
| **Encefalopatía espongiforme transmisible de primates no humanos** | Lemur y macaco *Rhesus* | 1996 |
| **Enfermedad priónica del camello** | Dromedario | 2018 |

**Tabla 2.** Enfermedades neurodegenerativas causadas por priones en humanos (modificada de Prusiner, 2013; Ironside et al., 2017).

| ENFERMEDAD | HOSPEDADOR | AÑO DE LA DESCRIPCIÓN |
|---|---|---|
| **Enfermedad de Creutzdfeldt-Jacob esporádico (eECJ)** | Humanos | 1920 |
| **Enfermedad de Creutzdfeldt-Jacob iatrogénica (iECJ)** | Humanos | 1974 |
| **Enfermedad de Creutzdfeldt-Jacob genético (gECJ)** | Humanos | 1995 |
| **Enfermedad de Creutzdfeldt-Jacob variante (vECJ)** | Humanos | 1996 |
| **Kuru** | Humanos | 1957 |
| **Insomnio familiar fatal** | Humanos | 1992 |
| **Insomnio fatal esporádico** | Humanos | 1999 |
| **Síndrome de Gerstmann-Sträussler-Scheinker (GSS)** | Humanos | 1936 |

De todas estas enfermedades incluidas en las tablas 1 y 2, en esta tesis doctoral nos vamos a centrar en el scrapie ovino por ser el prototipo de estudio del grupo de enfermedades priónicas. Además, es una enfermedad endémica en muchos países, lo que asegura el acceso a animales afectados de forma natural, además de no ser zoonótica, por lo que no exige las medidas de bioseguridad tan estrictas requeridas para el estudio en otras como la ECJ o la EEB.

## 2. SCRAPIE CLÁSICO OVINO

La enfermedad de scrapie (también denominada “tembladera “o “prúrigo lumbar”) es la enfermedad priónica mejor conocida y estudiada desde que fue reconocida por primera vez en el siglo XVIII en Europa (Inglaterra, 1732) (Gavier-Widen et al., 2005). Tiene la particularidad de ser la primera enfermedad priónica infecciosa y transmisible en condiciones naturales, siendo un grave problema en los rebaños a los que afecta. Hoy en día se distribuye en el mundo entero, a excepción de Australia y Nueva Zelanda, donde a pesar de que se describieron casos aislados importados de Reino Unido en los años 50, consiguieron erradicarlos y actualmente permanecen libres de scrapie (Bull and Murnane, 1958).

Aunque se trata de una enfermedad endémica en muchos países del mundo, los estudios sobre el modelo natural de la enfermedad de scrapie (van Keulen et al., 2000; González et al., 2005; Glaysser and Mabbot, 2007; Tabouret et al., 2010) han sido ampliamente superados por los que utilizan modelos experimentales. En la mayoría de los estudios recientes sobre patogénesis de las enfermedades priónicas se ha utilizado el modelo murino, tanto convencional como transgénico, además del de hámster (Ye et al., 1998; Beekes et al., 1998; Servida et al., 2007; Shah et al., 2017). Sin embargo, la extrapolación de resultados de este tipo de estudios experimentales no ha dado resultados tan exitosos como los esperados, ya que las conclusiones que se extraen a partir de ellos en ocasiones no reflejan la progresión real en los individuos afectados de forma natural por la enfermedad (Kriz et al., 2002; Gordon et al., 2007; Choi et al., 2010; Aisen et al., 2003). Por esta razón, actualmente existe un renovado interés en centrarse en el modelo natural. Por ello, los diversos aspectos considerados a lo largo de esta revisión bibliográfica se refieren al scrapie natural.

El desarrollo de esta patología va a estar determinado por dos factores importantes, como son la genética del hospedador y el agente casual o cepa de scrapie. También es crucial para tener una idea general de esta enfermedad neurodegenerativa conocer el curso patogénico, la sintomatología clínica asociada, los medios de diagnóstico disponibles y el posible potencial zoonótico.

### a. Influencia genética

En la oveja existen 3 localizaciones polimórficas importantes en el gen PRNP con influencia en la susceptibilidad al scrapie clásico: los codones 136, 154 y 171 (Goldmann et al., 1994). La valina (V) en el 136, arginina (R) en el 154 y glutamina (Q) en el 171 (haplotipo VRQ) aumentan esta susceptibilidad. Y al contrario, la alanina (A) en el 136, histidina (H) en el 154 y arginina (R) en el 171 (haplotipo AHR) confieren resistencia a ella. El haplotipo ARQ también es susceptible, mientras que el haplotipo ARR es el que mayor resistencia confiere frente al scrapie. En una minoría de razas se ha detectado, aunque con baja frecuencia, otro polimorfismo en lisina (K) en el 171. Las similitudes en cuanto a estructura sugieren que K puede comportarse parecido a R (Baylis and Goldmann, 2004).

Con el objeto de incrementar los genotipos resistentes en poblaciones ovinas (Goldmann, 2008) y así lograr erradicar los casos de scrapie clásico en Estados Unidos y la Unión Europea, se han aplicado programas de selección basados en el genotipado y sacrificio de animales con genotipos sensibles. Sin embargo, la prevalencia de la enfermedad continúa siendo elevada.

### b. Agente causal (cepas de scrapie)

Un concepto revolucionario en relación con las enfermedades priónicas es la existencia de diferentes agentes causales denominadas cepas, puesto que careciendo de material genético pueden comportarse de forma diferente y exhibir fenotipos e incluso vías patogénicas distintas (Sigurdson et al., 2007). La primera descripción de la existencia de cepas se publicó en 1961, cuando Pattison and Millson (1961) describieron diferentes "variantes" del scrapie tras haber transmitido scrapie ovino a cabras mediante inoculación intracerebral y haber observado distintos síndromes clínicos en la especie caprina, uno que denominaron *drowsy* (somnoliento) y otro *scratching* (rascado vigoroso). En aquella época, se desconocía la naturaleza del agente infeccioso y por ello emplearon el término "cepa", que se ha mantenido hasta la actualidad.

Las cepas, además de por los síntomas clínicos, como se acaba de indicar, pueden diferenciarse por los periodos de incubación y perfiles lesionales (Fraser and Dickinson, 1973), por la distinta morfología de los depósitos neuroanatómicos de $PrP^{sc}$ en varias regiones encefálicas evidenciados mediante inmunohistoquímica (IHQ) (Jeffrey and González, 2007; González et al., 2012) y/o por el perfil molecular en *Western Blot* (WB) (Collinge et al., 1996; Parchi et al., 1996). Mediante la técnica de IHQ, se ha logrado diferenciar más de doce patrones lesionales según el acúmulo y localización de la proteína prion (González et al., 2003).

Hasta el momento, se han aislado más de 20 cepas productoras de scrapie clásico en ovino y caprino (Dickinson and Fraser, 1972; Carp et al., 1987). Todas ellas poseen características moleculares, de transmisión y fenotípicas que se corresponden con la enfermedad endémica de una población (Biacabe et al., 2004), difiriendo de las de las cepas atípicas, con características moleculares y epidemiológicas distintas (Benestad et al., 2003).

Las interacciones entre la cepa y el hospedador han sido bien estudiadas (González et al., 2012), habiéndose descrito incluso la presencia de varias cepas de scrapie clásico en un mismo animal (Yull et al., 2006; Beck et al., 2012; Barrio et al., 2020). De hecho, la transmisión de 80 inóculos de scrapie en una misma línea murina transgénica mediante varios pases seriados permitió la identificación de al menos cuatro clases de agentes fenotípicamente distintos (Beringue et al. 2008; Thackray et al. 2012; Tixador et al., 2010).

### c. Patogenia

Existen dos vías principales de transmisión en ovino: placentaria y oral. En cuanto a la vía placentaria, se ha asociado un incremento en la incidencia de la enfermedad con la estación de cría, así que se piensa que la mayoría de animales se infectan al nacimiento o poco después

(Dickinson et al., 1965). Sería por contacto con priones presentes en los tejidos placentarios (cuya infectividad ha sido demostrada tanto en la oveja como en la cabra) (Onodera et al., 1993; Race et al., 1998; Schneider et al., 2015) o priones presentes en el ambiente (Saunders et al., 2012). Es decir, el mayor riesgo de transmisión natural sería de la madre al cordero con genotipos susceptibles a través de la placenta y los fluidos placentarios, resultando en una transmisión vertical. Además de una potencial transmisión horizontal a medida que el cordero crece (Touzeau et al., 2006). Recientemente ha sido demostrada la transmisión *in utero* durante las fases preclínica y clínica (Spiropoulos et al., 2014).

Por otro lado, la exposición oral al agente causal en un ambiente contaminado (van Keulen et al., 2002; Ersdal et al., 2005; Gough and Maddison, 2010; Mathiason, 2017) parece producirse mediante la ingestión, al cruzar la $PrP^{sc}$ la barrera intestinal al nivel del enterocito y pasar rápidamente a la linfa y la sangre, con las células M jugando un papel crucial en la captación del prion (Donaldson et al., 2012). La primera replicación del agente se produce en el tejido linfoide asociado al intestino, como las placas de Peyer (PP) del íleon (Andreoletti et al., 2000; Jeffrey et al., 2006). Se ha sugerido que la mayor cantidad de tejido linfoide asociado al intestino en animales jóvenes es un factor que probablemente incrementa la susceptibilidad en dichos individuos comparados con los más adultos (Press et al., 2004). La $PrP^{sc}$ se acumula también en las tonsilas y los nódulos linfáticos que las drenan, y después se disemina a otros tejidos linfoides (Andréoletti et al., 2000; van Keulen et al., 2008) durante meses antes de que la $PrP^{sc}$ se detecte en sistema nervioso central (SNC) (Race et al., 1992; Muramatsu et al., 1994). Durante esta fase preclínica o asintomática, las ovejas son fuente de contaminación ambiental, ya que se ha demostrado que excretan priones a través de la saliva (Tamgüney et al., 2012; Mathiason, 2017) y de las heces (Safar et al., 2008). La leche y el calostro representan un riesgo de transmisión adicional para las crías nacidas de ovejas afectadas (Konold et al., 2013) y también se ha demostrado la infectividad en sangre (Pattison and Millson, 1962; Dassanayake et al., 2015).

Para entender la patogénesis del scrapie es de vital importancia conocer cómo se produce la diseminación del agente causal desde órganos periféricos hasta el encéfalo de los individuos afectados. Aún no se sabe con seguridad cuál es el mecanismo por el que el prion pasa del sistema linforreticular (SLR) al sistema nervioso, pero diversos estudios sugieren que se produce desde las células dendríticas foliculares (CDF) hasta las fibras nerviosas que inervan los folículos linfoides (Bencsik et al., 2001; Heggebo et al., 2003). Se ha demostrado que el prion asciende del intestino a la médula espinal torácica y la médula oblongada u óbex a través

de los nervios esplácnico y vago. Desde estas regiones iniciales, se propaga craneal y caudalmente dentro del SNC (van Keulen et al., 2000).

La diseminación centrífuga desde el encéfalo a tejidos periféricos más lejanos parece ocurrir a través del sistema nervioso periférico (Andreoletti et al., 2004; Garza et al., 2014). De todos modos, la distribución tisular periférica de la $PrP^{sc}$ puede variar en función de varios factores, incluyendo la genética del hospedador, la dosis de $PrP^{sc}$ y la cepa de scrapie involucrada (Beekes, 2007; Garza et al., 2014).

**d. Sintomatología clínica**

Típicamente, el scrapie afecta a ovejas de entre 2 y 5 años (Dickinson et al., 1965; Georgsson et al., 2008) y a cabras de más de 6 años de edad (Colussi et al., 2008), no habiéndose evidenciado una afectación diferencial en función del sexo. El periodo de incubación varía entre 14 y 22 meses. La enfermedad presenta distintos síntomas nerviosos, de comportamiento y locomotores. El principal síntoma clínico del scrapie clásico es el prurito (por ello es también llamada prúrigo lumbar), provocando en el animal constantes lamidos, mordiscos y rascados con apoyo en las paredes y objetos, ocasionando alopecias cutáneas y heridas en zonas dorsal, lumbar, flancos, base de la cola, extremidades y cara (Vargas et al., 2006); pero también se incluyen otros síntomas como caquexia, temblores (de ahí el término "tembladera"), postura encorvada, ataxia de los miembros posteriores e hipermetría al andar (Dickinson, 1965). Además, los animales presentan frecuentemente alteraciones posturales, rechinar de dientes y temblores mioclónicos de cabeza, cuello y extremidades (Konold and Phelan, 2014a). El diagnóstico diferencial de dicha sintomatología incluye enfermedades como la listeriosis, toxemia o Visna Maedi.

De todas formas, hay que tener en cuenta que la sintomatología puede variar ampliamente entre animales debido a la diversidad de cepas causantes de la enfermedad (Collinge and Clarke, 2007). Las ovejas afectadas pueden vivir entre 1 y 6 meses tras la aparición de los síntomas clínicos, y el desenlace siempre es la muerte del animal.

Se puede distinguir el scrapie clásico del atípico por los síntomas clínicos (Konold and Phelan, 2014b). En este último, se observan signos de disfunción cerebelar, como ataxia e hipermetría más evidente, seguido de pérdida progresiva de condición corporal y comportamiento nervioso, habiendo ausencia de prurito (Konold et al., 2007).

e. **Diagnóstico**

El diagnóstico, como en el resto de enfermedades priónicas, sólo se puede confirmar con un examen *post-mortem* del encéfalo. Inicialmente se basaba en la presencia de vacuolización en el tronco del encéfalo mediante examen histopatológico tras tinción con hematoxilina eosina (HE). Según la Organización Mundial de Sanidad Animal (OIE), se recomiendan otros métodos basados en la detección de la $PrP^{sc}$ mediante WB o IHQ tras el tratamiento con proteinasa K que permiten diferenciar entre $PrP^{c}$ y $PrP^{sc}$ (OIE, 2019) para la confirmación de casos sospechosos.

La técnica de WB es ampliamente empleada (Katz et al., 1992; Madec et al., 2000; Mohri et al., 1992) porque además del resultado cualitativo (positivo/negativo), ofrece información de las características bioquímicas de la $PrP^{sc}$ y, con ello, permite la diferenciación de cepas. El patrón de glicosilación en WB, definido como la proporción relativa de las tres glicoformas de la proteína, es único de cada cepa priónica.

Para IHQ, se utilizan secciones del tronco del encéfalo a nivel del óbex (Wood et al., 1997), que es la primera área del encéfalo inmunorreactiva en casos preclínicos de scrapie clásico (van Keulen et al., 2008). No así de scrapie atípico, en el que el cerebelo es la región encefálica más afectada mientras que en óbex se observan cambios mínimos (Benestad et al, 2008; Simmons, et al., 2010). También del tronco del encéfalo a nivel de pedúnculos cerebelares y mesencéfalo (OIE, 2019).

Hay un problema relativo a ambas pruebas de detección de $PrP^{sc}$ puesto que no existe un protocolo común para todos los laboratorios de referencia, a pesar de que se ha observado que la resolución de las técnicas puede variar en función del estado de la muestra, los pretratamientos o los anticuerpos utilizados (Hardt et al., 2000; Monleón et al., 2004).

En los programas de vigilancia activa de grandes poblaciones se han utilizado tests rápidos (*screening*) con el objeto de analizar los animales destinados al consumo humano; dado que el depósito de $PrP^{sc}$ precede a la vacuolización y a la aparición de signos clínicos, dichos tests son la opción más rápida (Hamir et al., 2001).

Por otro lado, el único método diagnóstico que existe para la detección de $PrP^{sc}$ *in vivo* es la realización de biopsias de tejido linfoide accesible: tercer párpado (O'Rourke et al., 2000), tonsila (Schreuder et al., 1998) o mucosa rectal (Gavier-Widén et al., 2005; González et al., 2005), combinado con la observación clínica (Gough et al., 2015). A pesar de que se trata de una herramienta muy útil para la detección de casos preclínicos con un 100 % de especificidad,

su sensibilidad no es tan alta puesto que se basa en la presencia detectable de $PrP^{sc}$ en tejidos linfoides periféricos bastante antes de que sea aparente en el encéfalo y médula espinal (van Keulen et al., 2000). Pero como se ha indicado con anterioridad, ésta depende de varios factores como el genotipo del hospedador o la cepa causal.

**f. Potencial zoonótico**

El scrapie se ha considerado tradicionalmente como una enfermedad no zoonótica debido a que todos los estudios epidemiológicos publicados no establecen una relación causal entre el scrapie ovino y las infecciones en humanos (Chatelain et al., 1981). Además, no ha sido posible demostrar la transmisión del scrapie a primates no humanos (Gibbs and Gajdusek., 1971) ni a ratones transgénicos que sobrexpresaban la proteína priónica humana (Wadsworth et al., 2013). Existe un estudio experimental relativamente reciente con resultados conflictivos acerca de este potencial zoonótico, al haber demostrado la capacidad de algunos agentes para cruzar la barrera de transmisión humana, utilizando para ello modelos experimentales con ratones transgénicos humanizados (Cassard et al., 2014).

Puesto que también se ha evidenciado la presencia de priones en músculo esquelético de animales infectados de forma natural (Andreoletti et al., 2004; Garza et al., 2014), su potencial zoonótico a través del consumo debería de ser caracterizado completamente (Houston and Andreoletti, 2018), por lo que se necesitan más estudios para evaluar el riesgo real que las cepas de scrapie clásico pueden llegar a tener para la salud pública (Greenlee, 2019).

Aunque la patología de las enfermedades priónicas parece estar restringida al SNC, algunas de ellas afectan a tejidos linfoides secundarios del sistema inmune del hospedador (tonsilas, nódulos linfáticos, etc) antes de la neuroinvasión. En el siguiente capítulo se justifica por qué dicho sistema inmune resulta relevante para la patogénesis de la enfermedad de scrapie.

A pesar de que originalmente se asumió que en las enfermedades priónicas no existía respuesta inmunológica debido a la ausencia de respuesta inmune humoral a la $PrP^{sc}$ y a la falta de producción de interferón en el hospedador infectado (Riesner, 2007), la respuesta del hospedador es central en la patogénesis de las enfermedades priónicas y en concreto del scrapie (Mabbot et al., 1998). De hecho, en los últimos años, ha surgido la hipótesis de la neuroinflamación o inflamación del SNC para dar un nuevo enfoque que justifique el proceso de degeneración común en todas las patologías neurodegenerativas, incluidas las priónicas.

## 3. INMUNOPATOLOGÍA DEL SCRAPIE

Hasta hace poco el SNC estaba considerado como una entidad "inmunoprivilegiada" que carecía de un sistema de drenaje linfático (Aspelund et al., 2015; Louveau et al., 2015) y que además está protegido por la barrera hematoencefálica (BHE), una vasculatura especializada que consiste en células endoteliales capilares unidas por uniones finas. Como resultado, la BHE es impermeable a la mayoría de sustancias solubles de tamaño grande, incluyendo inmunoglobulinas, restringiendo la migración de células linfoides dentro del SNC (Carvey et al., 2009). A su vez, los capilares del SNC están casi completamente rodeados por pies terminales astrocíticos, por lo que el astrocito contribuye a la integridad estructural de la BHE. Y esta BHE, junto con las neuronas y la microglía, constituyen una estructura llamada "unidad neurovascular" (Hawkins and Davis, 2005), por lo que existen interacciones entre estos tipos celulares que regulan el flujo vascular de entrada al SNC.

Un hecho destacable es que la $PrP^{c}$ se expresa en linfocitos T y B, células natural killer, plaquetas, monocitos, células dendríticas y foliculares (Aguzzi and Heikenwalder, 2005). Esta expresión celular ubicua tiene importantes consecuencias inmunológicas, ya que la $PrP^{sc}$ es tolerada por el sistema inmune del hospedador, previniendo el desarrollo de respuestas inmunes específicas frente a ambas proteínas, celular y patológica. Probablemente como consecuencia de ello, no se han descrito respuestas inmunes específicas en animales afectados de enfermedad priónica, a pesar de que existan altos niveles de priones en los órganos linfoides secundarios (Tsukamoto et al., 1985; Mabbot et al., 2018). Este patrón de expresión junto con la falta de respuesta inmune a un autoantígeno ha llevado a pensar que el sistema inmune juega un papel crucial en la replicación periférica y asintomática del prión, así como en su acceso al SNC, asociado con las manifestaciones clínicas de la enfermedad (Aucouturier et al., 2000; Wisniewski and Goni, 2010).

Algunas células inmunes particularmente importantes para esta replicación periférica son las células dendríticas foliculares (CDF) y las células dendríticas migratorias derivadas de la médula ósea (Aucouturier et al., 2001; Beekes and McBride, 2007; Langevin et al., 2010). Ya se sugirió que en el intervalo entre la exposición del animal a la proteína patológica y la neuroinvasión, las CDF son potenciales dianas de intervención terapéutica (Brown et al., 1999), demostrándose además que la replicación del agente causal del scrapie en tejidos linfoides depende de la $PrP^{c}$ que expresan las CDF (Brown et al., 1999). Se ha sugerido que las CDF podrían transportar priones desde su sitio de replicación hasta nervios periféricos en órganos linfoides, permitiendo así el proceso de neuroinvasión (Agguzi, 2006). Experimentos que

utilizaban ratones transgénicos con ausencia de expresión de CDF evidenciaron la replicación en otras células en los folículos linfoides (Prinz et al., 2002). Por lo tanto, células inmunes poco definidas o posiblemente células precursoras mesenquimales (como las CDF) podrían ser capaces también de replicar el agente infeccioso (Oldstone et al., 2002; Prinz et al., 2002).

Por el contrario, durante la progresión de la patología se activa la respuesta inmune innata, particularmente llevada a cabo por fagocitos tales como macrófagos, microglía o células dendríticas, que son movilizados para ingerir y degradar la $PrP^{sc}$ reduciendo así la carga priónica (Beringue et al., 2000; Marella and Chabry, 2004).

Los linfocitos y las células B también tienen un cierto papel inmunopatológico, ya que los priones también se han detectado en asociación con linfocitos (Andreoletti et al., 2012; Douet et al., 2016) y células B en sangre de ovejas afectadas de scrapie (Edwards et al., 2010). Además, la mayoría de agentes causales de la enfermedad se replican en bazo y linfonodos antes de la neuroinvasión (Kimberlin and Walker, 1979). En varios estudios se ha demostrado que la inmunosupresión mediante esplenectomía o administración de fármacos inmunosupresores, incrementa la duración del periodo de incubación (Aucouturier et al., 2000), mientras que la inmunoestimulación inespecífica tiene el efecto opuesto (Bremer et al., 2009). Una estrategia similar ha sido llevada a cabo en pacientes y modelos animales de Enfermedad de Alzheimer (EA) (Wisniewski and Sigurdsson, 2010; Ostrowitzki et al., 2012;), demostrando que mejoraban esta patología (Morgan, 2011).

Sin embargo, el efecto potencialmente beneficioso que tendría el hecho de alterar la respuesta inmune del hospedador a la $PrP^{sc}$ debería evaluarse para cada cepa específica, tanto en el SNC como en el periférico, ya que diferentes cepas de scrapie tienen como diana diferentes poblaciones celulares en los tejidos linfoides, al igual que ocurre en el SNC.

Además, se conoce que las condiciones inflamatorias periféricas inducen el acúmulo y replicación de priones en órganos previamente considerados como libres de infección. Para investigar si el proceso inflamatorio influía en la patogénesis del prion, se inoculó la cepa RML en modelo murino con patologías inflamatorias del riñón, páncreas e hígado. En todos los casos y órganos estudiados, la inflamación linfocítica crónica resultó en una acumulación de proteína en órganos que de otra forma estarían libres (Heikenwalder et al., 2005). También se ha observado que en animales de granja existe depósito de prion en glándulas mamarias afectadas de mastitis (Ligios et al., 2005). Además, se ha evidenciado una relación similar en ovejas afectadas de scrapie y Maedi Visna conjuntamente, en las que se halló presencia de priones en órganos junto a infiltrados inflamatorios linfocitarios típicos de la enfermedad de

Maedi Visna (Salazar et al., 2010). Las consideraciones que esto conlleva para la salud pública hacen pensar que deberíamos incrementar nuestro conocimiento sobre mecanismos subyacentes, especialmente los relacionados con la inflamación.

Por otro lado, Romero-Trevejo et al., 2010 realizaron un estudio inmunohistoquímico para valorar la presencia de macrófagos y la dinámica de citoquinas en el intestino de ratones con scrapie oralmente infectados con la cepa RML. Los resultados apuntaron a un posible papel de los macrófagos en la captación y transporte del agente infeccioso a la Placa de Peyer. El estudio sobre citoquinas indicó la presencia de una respuesta dañada o perjudicada, mediada por células Th1 y Th2 que podría facilitar la propagación del prion al SNC.

Todo lo anteriormente citado constituye parte de la inmunopatología durante el periodo crítico, que es la larga fase de incubación asintomática o fase preclínica, en la que el prion se replica periféricamente. En esta fase, diversos agentes terapéuticos, que no cruzan la BHE, podrían tener posibilidades de éxito en el tratamiento de la enfermedad (Mabbot et al., 2018).

Con respecto al proceso que sucede tras la neuroinvasión, se ha descrito el incremento de una gran batería de citoquinas proinflamatorias y quimioquinas en el SNC en respuesta a la presencia de prión, mayoritariamente liberadas por los astrocitos y la microglía (Williams et al., 1994a, 1997a). Este hecho indica la activación de ambas poblaciones gliales. Williams et al., 1997a, mostraron que la activación de la microglía sucede a la formación de placas de $PrP^{sc}$ y precede a la vacuolización y muerte neuronal. Estos datos refuerzan las observaciones de otros estudios sobre scrapie murino (Campbell et al., 1994; Kordek et al., 1996; Williams et al., 1997b), confirmando que las respuestas inflamatorias locales del SNC están mediadas por la microglía reactiva en las etapas finales de la enfermedad. Además, también se ha demostrado que la astroglía está altamente implicada en scrapie (Chesebro et al., 2005; Sarasa et al., 2012; Hernández et al, 2014; Hollister et al, 2015).

Por lo tanto, a día de hoy se mantiene que la neuroinflamación implica un papel relevante de estas células gliales del SNC, ya que la infiltración de lecucocitos de la periferia es limitada y poco detectable, tan sólo en los últimos estadios de la enfermedad clínica (Williams et al., 1995; Carroll et al., 2016). Así, está aceptado que las enfermedades priónicas tienen un componente neuroinflamatorio que puede jugar un papel clave en la neurodegeneración Crespo et al., 2012; Aguzzi et al., 2013; Heneka et al., 2014).

En relación con esta respuesta glial, en el estudio de Kang et al., 2016, se utilizaron cultivos primarios mixtos de neuronas y glía, demostrando que en estadios tempranos las células gliales responden a través de la inmunidad innata mediada por receptores tipo *Toll* (TLR). En

presencia de proteína prion recombinante, las células gliales sobrerregularon la expresión de TLR tipos 1 y 2 y secretaron factor de necrosis tumoral alfa (TNF-α), interleuquina 1 beta (IL-1β), interleuquina 6 (IL-6) e interferón gamma (IFN-γ). Concluyeron que los TLR que median las respuestas inmunes inhiben la propagación del prion. Posteriormente, se ha descrito que concretamente el TLR2 confiere neuroprotección parcial durante la enfermedad (Carroll and Chesebro, 2019).

En resumen, se han realizado avances importantes durante las últimas cuatro décadas respecto al entendimiento del papel del sistema inmune en las enfermedades priónicas. Sin embargo, a pesar de todo el progreso conseguido, existen muchos enigmas que aún se desconocen (Requena et al., 2016).

Como se ha citado que las células de la neuroglía juegan un papel central en el proceso inmunopatológico de las enfermedades priónicas, se incluye a continuación una revisión sobre ella.

### a. Papel de la neuroglía en las enfermedades neurodegenerativas

Las células gliales se clasifican en dos grandes grupos: macroglía y microglía. La macroglía agrupa a su vez cuatro tipos de células especializadas: células ependimales, células de Schwann, oligodendroglía y astroglía (García-Marín, 2007). Esta última incluye astrocitos, glía marginal, glía radial del encéfalo en desarrollo y médula espinal, células de Bergmann en el cerebelo, células de Müller en la retina, pituicitos en la neurohipófisis y tanicitos en el hipotálamo (García-Segura and McCarthy, 2004).

La activación de las células gliales, evidenciable mediante la hiperplasia y/o hipertrofia astroglial y cambios morfológicos y de expresión de marcadores microgliales es lo que se denomina gliosis. Su posible implicación en la neuroprotección y/o neurotoxicidad constituye un reto a aclarar en el contexto de la hipótesis de la neuroinflamación.

#### i. Astroglía

Los astrocitos son las células gliales más numerosas, de origen ectodérmico y neuroepitelial y derivan de la glía radial (Kriegstein and Alvarez-Buylla, 2009).

En un principio, esta población glial se clasificó en dos tipos de astrocitos, teniendo en cuenta la morfología, el número de procesos y su localización: fibrosos (los que presentaban menor cantidad de procesos pero más largos y se encontraban mayoritariamente en la sustancia blanca), y protoplasmáticos (con una mayor cantidad de procesos más cortos, localizados en la sustancia gris) (García Marín 2007; Kettenmann and Verkhratsky, 2008). Sin embargo,

actualmente, la clasificación astrocitaria incluye varias subpoblaciones celulares con morfologías y funciones muy diferentes (Verkhratsky and Nedergaard, 2018): astrocitos fibrosos, protoplasmáticos, velados (variante morfológica de los protoplasmáticos, con pequeño soma y procesos cortos en forma de hoja), de Gomori (localizados principalmente en hipotálamo e identificados con una tinción específica de Gomori), asociados a superficie (localizados en el córtex posterior prefrontal y amigdaloide en humanos), marginales o perivasculares (próximos a la piamadre, formando numerosos pies vasculares), interlaminares (de pequeño tamaño y localizados en la corteza cerebral de primates y humanos exclusivamente), polarizados (localizados sólo en la corteza cerebral humana), astroglía radial (o glía radial adulta) y glía radial (células neurales precursoras universales, que sólo aparecen durante el desarrollo cerebral). Dicha clasificación requiere el análisis de varios marcadores inmunohistoquímicos y moleculares (Verkhratsky and Nedergaard, 2018), siendo el principal utilizado para su identificación genérica la proteína acídica glial fibrilar (GFAP), expresada tanto por astrocitos maduros, como *de novo* (Bignami et al., 1972).

Los astrocitos cumplen varias funciones esenciales Verkhratsky and Nedergaard, 2018):

- Controlan el ambiente en el encéfalo y regulan la plasticidad neuronal, dando apoyo metabólico y trófico (Catorce and Gevorkian, 2016).

- Sirven de sostén mecánico, definiendo la arquitectura de la sustancia gris, estando presente en todas las capas del SNC, incluso las más externas, y formando las capas piales.

- Participan en el impulso eléctrico, formando parte de las redes neuronales mediante la sinapsis tripartita, protegiendo a las neuronas mediante la secreción de neurotrofinas (Lindholm et al., 2007) o antioxidantes (Shih et al., 2003), entre otros.

- Controlan el balance energético y la síntesis de glucógeno para suministrar energía a las neuronas.

- Participan en la fagocitosis de la sinapsis durante el proceso de remodelación neuronal del SNC (función que hasta hace poco era atribuida solamente a la microglía) (Chung et al, 2013).

- Forman parte de la BHE mediante los pies astrocíticos terminales, jugando un papel en el tráfico metabólico cerebral entre neuronas y vasos sanguíneos intracerebrales (Kacem et al., 1998).

- Parecen regular el transporte de agua a través de sus pies, al expresar acuaporina 4 de forma polarizada en los terminales astrocíticos (Satoh et al., 2003).

- Participan en la neurogénesis a través de la glía radial. También en la neurogénesis adulta.

- Participan en funciones defensivas del SNC, teniendo como objetivo restablecer la homeostasis tisular tras el daño (Pekny et al., 2016).

Cuando se daña el SNC, los astrocitos responden activándose. Esta reacción, conocida como astrogliosis es el resultado de la proliferación de astrocitos, cambios morfológicos dentro de la célula (por ejemplo, hipertrofia) y aumento de la expresión de marcadores como GFAP (Norenberg, 1996; Sofroniew and Vinters, 2010), la glutamina sintetasa o S100b (Noremberg, 1979; Goncalves et al., 2008), aunque estos últimos no son exclusivos de astrocitos. Estudios recientes han llevado a cabo análisis genéticos del transcriptoma astrocítico a gran escala en muestras murinas y humanas, identificando muchas moléculas en gran cantidad en los astrocitos diferenciales de otras células como neuronas u oligodendrocitos (Lovatt et al., 2007; Cahoy et al., 2008). En general, intentan investigar moléculas candidatas potenciales para identificar mejor los astrocitos. Una de estas moléculas parece ser la proteína ALDH1L1, cuya identificación mediante inmunohistoquímica puede llegar a ser un marcador sensible para la mayoría de los astrocitos (sino todos) en el tejido encefálico sano (Barres, 2008).

Aunque la activación fisiológica de los astrocitos es un mecanismo protector importante en el encéfalo, la activación incontrolada de estas células gliales puede conllevar efectos perjudiciales, dando lugar a la liberación de mediadores inflamatorios e incluso neurodegeneración (Bronzuoli et al., 2016; Norden et al., 2016). Existen estudios que han demostrado que los astrocitos pueden estar implicados en procesos inmunológicos e inflamatorios que ocurren dentro del encéfalo (Farina et al., 2007). Se ha encontrado (tanto *in vitro* como *in vivo)* que varias citoquinas, como IL-1, TNF-α e IL-6 (Giulian et al., 1986; Selmaj et al., 1990), están implicadas en la proliferación astrocítica, y, una vez que se activa este tipo glial, puede ser inducido para expresar antígenos del complejo mayor de histocompatibilidad 1 y 2 (MHC I y II) y secretar más citoquinas.

En el caso de las enfermedades neurodegenerativas, la mayoría de ellas implican proliferación de astrocitos, y aunque no es indicativa de cambios específicos en el SNC, estos cambios suelen ir acompañados de daño o muerte neuronal (Pekny and Pekna, 2016). En el caso de las enfermedades priónicas, es una de las características neuropatológicas genuinas, tal y como se

enunciaba al inicio de esta revisión (Pekny and Nilsson, 2005). Se ha demostrado que junto con las neuronas, los astrocitos son localizaciones importantes donde sucede la replicación de la proteína prion en modelos experimentales (Race et al., 1995; Raeber et al., 1997). Lo que no queda claro todavía es el orden en el que acontece la astrogliosis y la neurodegeneración. Mientras que algunos autores proponen que la activación de las células gliales precede a la muerte neuronal en modelos murinos de scrapie (Williams et al., 1994a, 1997a; Riemer et al., 2000), otros indican lo contrario (Lasmezas et al., 1996). En el modelo de scrapie en hámster inoculado vía intracerebral se observó que el acúmulo de $PrP^{sc}$ precedía al de expresión de GFAP, que aumentaría a los 5 ó 15 días (Jendroska et al., 1991; Lasmezas et al., 1996; Brown and Monhn, 1999; Brown, 1999). Todos estos hallazgos apoyan la relación causa – efecto entre el acúmulo de la proteína prion y el desarrollo de astrogliosis y lesiones cerebrales, pero queda todavía sin determinar cuál es la causa y cuál el efecto.

Nuestro grupo de investigación ha logrado avances en relación con esta idea, demostrando que los astrocitos están implicados tanto en la progresión del scrapie natural ovino (Sarasa et al., 2012; Hernández et al., 2014), como en enfermedades priónicas humanas (Monzón et al., 2018) y prion-*like* (Garcés et al., 2019).

Recientemente, utilizando cultivos celulares, se han descrito astrocitos neurotóxicos (llamados astrocitos A1) que se consideran contribuidores potenciales de la muerte de neuronas y oligodendrocitos en varias enfermedades neurodegenerativas (Liddelow et al., 2017; Liddelow and Barres, 2017). Actualmente, este subtipo de astrocitos y no la microglía, podrían estar implicados en la muerte neuronal hasta un mayor punto del que se pensaba previamente, habiéndose sugerido que las citoquinas son las implicadas en el cambio de papel de los astrocitos de neuroprotectores a neurotóxicos (Efremova et al., 2017). A pesar de que resulta totalmente necesaria la presencia de microglía para que los astrocitos presenten este fenotipo activado (Kirkley et al., 2017).

De esta forma, los astrocitos tienen una doble función muy peculiar, siendo tanto la mayor población neuroprotectora en el encéfalo sano como también unas células potentemente inflamatorias que pueden ejercer efectos dañinos en situaciones de enfermedad (Allen and Barres, 2009; Sofroniew and Vinters, 2010), comprometiendo la supervivencia neuronal (Buffo et al., 2010).

La interrelación entre astrocitos y microglía es evidente y cada vez cobra más importancia a medida que se reconoce un papel más relevante en el desarrollo de la neurodegeneración en general y la enfermedad priónica en particular (Kranich et al., 2010; Aguzzi and Zhu, 2017). Así,

la activación microglial y la astrogliosis deben estar más relacionadas de lo que previamente se pensaba, y se requieren más investigaciones en esta línea debido a la diversidad de subtipos de astrocitos que existen en el SNC.

### ii. Microglía

Las células de la microglía constituyen aproximadamente un 10 % de la población glial total y se considera que son los macrófagos residentes en el encéfalo (Perry and Gordon, 1988). Son unas células extremadamente plásticas y tienen muchas ramificaciones en el estado latente o de reposo, a través de las cuales están vigilando el ambiente constantemente. Los avances en el estudio de esta población glial comenzaron hace más de 100 años, en 1913, cuando fueron descritas por Cajal como el "tercer elemento" del SNC, después de las neuronas y los astrocitos (Garden and Möller, 2006; García-Marín et al., 2007). Más tarde, en 1918, su discípulo Pío del Río Hortega publicó un método para teñir estas células microgliales y distinguirlas de las células colindantes del SNC (Río Hortega, 1918; Hickman et al., 2018). Estudios ontológicos sobre la microglía confirmaron las sospechas de Hortega de que son células mesenquimales, mieloides (Kierdorf et al., 2013), originadas en el saco vitelino y con capacidad de autoregeneración independiente de las células madre hematopoyéticas (Tay et al., 2017). En los años 80 fue posible visualizar este tipo glial por primera vez usando técnicas inmunohistoquímicas (Streit et al., 2000) y pronto se llegó a la conclusión de que la microglía produce un gran número de sustancias mediadoras potencialmente tóxicas (Banati et al., 1993). La primera evidencia de que la actividad de la microglía intacta era esencial para la homeostasis del encéfalo vino a través del experimento de Bianchin et al., 2004, en el que se utilizó como modelo una enfermedad neurodegenerativa extremadamente rara (llamada enfermedad de Nasu-Hakola, que es un tipo de demencia que conduce a la muerte de personas jóvenes menores de 50 años).

No existen antígenos específicos de microglía, ya que todos los marcadores fenotípicos (como CD68 o MHC II) son compartidos con otros tipos celulares tipo macrófagos. Solamente se puede identificar la microglía mediante antígenos de superficie celular, entre los que figuran los receptores Fc de inmunoglobulinas (Perry et al., 1985; McGeer et al., 1993).

Como se ha indicado, la microglía en reposo tiene un fenotipo ramificado. Cuando existe daño tisular o invasión por algún patógeno en el SNC, la primera respuesta de la microglía es la migración al sitio lesionado, como resultado de la segregación de factores quimiotácticos que estimulan esta migración; además, el número de células microgliales se incrementa mediante proliferación *in situ* (Sedgwick et al., 1998) o reclutamiento de monocitos circulantes en

sangre, que después sufren una transición a forma ameboide tras su activación (Ransohoff and Perry, 2009) que está asociada con la expresión de genes neuroinflamatorios (Kim and de Vellis, 2005). Así, los fenotipos que estas células macrofágicas pueden adoptar según el estado de activación se resumen en (Tang and Le, 2016; Kempuraj et al., 2016):

- Macrófagos desactivados, como respuesta a citoquinas antiinflamatorias, como la IL-10 (fenotipo en reposo, ramificado) (Streit, 2002).

- Activación clásica o M1, como respuesta proinflamatoria al IFN-γ producida por linfocitos Th1 o señalización a través de los TLR, en la que se liberan IL-1β, IL-6 y TNF-α.

- Activación alternativa o M2, como respuesta antiinflamatoria asociada con neuroprotección (Colton, 2009), en las que los linfocitos Th2 producen IL-4, IL-10 e IL-13.

A su vez, la clasificación morfológica de esta población glial a lo largo de su proceso de activación incluye (Boche et al., 2013): microglía ameboide (morfología esférica, asociada a situación de fagocitosis), distrófica (asociada a la edad), *rod-like* (forma de bastón, asociada a enfermedades crónicas), células multinucleadas (asociada a reacción fagocítica de material no digerible) y macrófagos epitelioides (*cluster* con forma de granuloma asociado a infecciones crónicas).

También se han detectado cambios morfológicos en la microglía asociados a la edad y al género en humanos (Nissen, 2017). Un estudio *in vivo* reciente muestra que la microglía exhibe un fenotipo asociado a la edad que presenta un volumen incrementado del soma, procesos más cortos y una distribución tisular más heterogénea, además de que la respuesta a la lesión está disminuida con la edad (Hefendehl et al., 2014). Por lo tanto, la edad por sí misma también puede ser responsable de un funcionamiento anormal de la microglía.

Las funciones principales que ejerce la microglía son (Ginhoux and Prinz, 2015):

- Fagocita detritus celulares (célula "carroñera"). Además de ser importante para la remodelación de tejidos en el SNC en desarrollo (Perry and Gordon, 1988), elimina células dañadas o apoptóticas en edad adulta, restos celulares y diversos microorganismos. Esta función defensiva está relacionada con el resto de funciones (Boche et al., 2013) que se describen a continuación.

- Participa en la neurogénesis y remodelación nerviosa en adultos (Sato, 2015).

- Participa en el mantenimiento y regulación de la BHE (Abbott et al., 2006)

- Está implicada en las respuestas inmunes en el SNC. Actúa como célula presentadora de antígenos, ya que expresa diferentes antígenos de superficie, entre ellos los antígenos del MHC una vez activada la célula (McGeer et al., 1993), secreta citoquinas inmuno-reguladoras y responde a la vez a la estimulación con citoquinas.

- Participa directamente en la sinapsis junto con astrocitos y neuronas (Kettenmann et al., 2013).

- Interactúa con los astrocitos en los procesos de homeostasis e inflamación. Se ha sugerido que, por lo tanto, posiblemente en la neurodegeneración (Lidelow et al., 2017), ya que, como se ha indicado anteriormente, la microglía es esencial para que los astrocitos expresen un fenotipo activado (Kirkley et al., 2017).

Por ello, la activación microglial, junto con la astroglial, representa una de los hechos distintivos de muchas enfermedades neurodegenerativas (Heneka et al., 2014), considerándose actualmente que contribuye al proceso neuroinflamatorio (Vincenti et al., 2015), ya que su activación crónica y desregulada puede conducir a un tono inflamatorio que dé lugar a un malfuncionamiento y daño de las células nerviosas (Rea et al., 2016). Un factor clave es que la microglía nunca está en reposo total; sus funciones sensoras y protectoras las mantienen constantemente activadas. La desregulación de cualquiera de esas funciones resulta en un desequilibrio que inicia o propaga la neurodegeneración (Hickman et al., 2018). Determinar la contribución relativa de la proliferación y activación de las células microgliales es esencial para entender el desarrollo de estas patologías (Ponomarev et al., 2005). Por lo tanto, analizar el grado de proliferación microglial bajo condiciones patológicas es clave para entender cómo la respuesta inflamatoria innata contribuye al inicio y progreso de la enfermedad.

En las patologías priónicas, las células microgliales se encuentran activadas, lo cual viene definido por su morfología alterada y la sobreexpresión de marcadores de superficie celular (Betmouni et al., 1996). Los estudios de Williams et al., 1994a fueron los primeros que notificaron cambios en la microglía en modelos de este grupo de enfermedades. De hecho, la presencia de microglía activada adyacente a los depósitos de $PrP^{sc}$ es una característica común en los encéfalos afectados tanto de humanos como de animales (Guiroy et al., 1994; Williams et al., 1997a). Dicha activación es detectable desde estadios iniciales (Betmouni et al., 1996), y se va propagando a medida que la enfermedad avanza, estrechamente relacionada con el

proceso de la neurodegeneración (Perry, 2016). Se ha determinado que la distribución espacial de microglía activada está más correlacionada con áreas de degeneración sináptica (Cunningham et al., 2003) y vacuolar (Williams et al., 1994a, b) que con el propio acúmulo de $PrP^{sc}$.

Aun no se sabe cómo ocurre el daño neuronal, pero hay evidencias en modelos experimentales de patología priónica que apuntan a que la microglía tiene un papel crucial, ya que su acúmulo y activación van paralelos al patrón espacial y temporal de depósito de $PrP^{sc}$, lo cual va seguido de vacuolización y muerte neuronal (Betmouni et al., 1996; Williams et al., 1997a), que es la causante del inicio de los síntomas clínicos de la enfermedad. De hecho, la implicación de la microglía en la muerte neuronal está bien descrita en un gran número de enfermedades del SNC, y entre ellas las enfermedades priónicas (Brown, 2001; Heppner et al., 2001), siendo asociada la mayoría de la respuesta inflamatoria de este grupo de enfermedades a dicha activación microglial (Vincenti et al., 2015). En los estudios de Brown et al., 1996 y Giese et al., 1998 se demostró (mediante un modelo *in vitro* usando un fragmento de prion P106-126) que para que haya neurotoxicidad se requería la presencia de microglía activada. De hecho, en un principio se propuso que podía ser un sitio de replicación para los priones, debido a que la microglía de encéfalos infectados exhibía altos niveles de infectividad (Baker et al., 1999). Además, está bien establecido que esta población glial es capaz de fagocitar neuronas apoptóticas (Hughes et al., 2010; Kranich et al., 2010) y que la función fagocítica de la microglía se incrementa en encéfalos afectados de enfermedad priónica. Hay experimentos que han demostrado claramente que las células de la microglía activada ejercen sus efectos neurotóxicos mediante una estimulación de la muerte celular por vía apoptótica incluso en ausencia de contacto directo entre neuronas y microglía (Marella and Chabry, 2004).

Aunque la microglía fagocite la $PrP^{sc}$ durante el inicio de la enfermedad (fase preclínica y clínica inicial, activación M2), parece que dicha fagocitosis es insuficiente cuando existe un acúmulo sostenido de $PrP^{sc}$ que conduce a daño neuronal (fase clínica establecida). Este hecho podría hacer que la microglía cambie a un fenotipo proinflamatorio (activación M1) y al final acabe ejerciendo efectos perjudiciales para el SNC (Hughes et al., 2010; Aguzzi and Zhu, 2017). Por lo tanto, una estrategia para combatir las enfermedades priónicas y prion-*like* podría incluir la búsqueda de terapias que reprogramen las respuestas microgliales para alejarse de las respuestas proinflamatorias y dirigirlas hacia mecanismos de eliminación proteica.

En resumen, se han propuesto numerosos estadios de activación microglial (Streit et al., 1999; Nelson et al., 2002; Mathys et al., 2017). Determinar si esas subpoblaciones exhiben

características neuroprotectoras y/o neurotóxicas aportarían un gran avance en el estudio, tanto de la enfermedad priónica como las prion-*like.*

Puesto que se ha citado la aparente implicación de las citoquinas liberadas por ambos tipos de células gliales (astrocitos y microglía) en la patología neurodegenerativa, se realiza una exhaustiva revisión sobre la bibliografía relacionada en este tema.

### b. Implicación de las citoquinas en las enfermedades neurodegenerativas

Las citoquinas son proteínas de bajo peso molecular que ejercen el papel de mensajeros químicos entre las células del sistema inmune. En condiciones fisiológicas, los niveles de citoquinas se mantienen a niveles bajos (Pitossi et al., 1997). Por el contrario, si existen condiciones patológicas, los niveles se elevan hasta 100 veces (Kim et al., 2016). Están generalmente asociadas con inflamación, activación inmune y diferenciación o muerte celular, e incluyen interleuquinas (IL), interferones (IFN), factor de necrosis tumoral (TNF), quimioquinas y factores de crecimiento (Alan and Rothwell, 2001). Aunque originalmente fueron conocidas por su habilidad para regular la actividad de los leucocitos durante las respuestas inflamatorias, actualmente se sabe que estas pequeñas proteínas ejercen múltiples funciones (mantenimiento de la homeostasis, reparación de tejidos y regeneración) y están producidas por diferentes tipos celulares (Wang et al., 2002), entre los que se encuentran las células de la neuroglia (John et al., 2003). La mayoría de las citoquinas son producidas en la periferia y pueden actuar tanto sistémica como centralmente, donde activan vías neurales de la respuesta inmune como el eje hipotálamo-pituitaria-adrenal (HPA), constituyendo así una señal reguladora entre el sistema inmune y el SNC (Benveniste, 1992).

Generalmente, las citoquinas se clasifican en dos grupos: proinflamatorias y antiinflamatorias, que facilitan o inhiben las respuestas inflamatorias, respectivamente. Dentro del primer grupo se encuentran algunas como la IL-1β, IL-6 y TNF-α, mientras que en el segundo se encuadran la IL-4 y la IL-10 (Lee and Chau, 2002). En general, las citoquinas antiinflamatorias, como la IL-10, promueven la supervivencia neuronal, mientras que las proinflamatorias, como el TNF-α, pueden inducir su muerte.

Los efectos de las citoquinas y otros factores de señalización inflamatoria son pleiotrópicos (la mayoría llevan a cabo numerosas funciones), redundantes (se llevan a cabo por duplicado) y cooperativos (algunas acciones específicas dependen de la presencia o ausencia de otras citoquinas) (Hueston and Deak, 2014). Además, hay que tener en cuenta que los efectos dañinos de una citoquina pueden conllevar acciones beneficiosas para el organismo (Planas et

al., 2006). Por ejemplo, en modelos de isquemia cerebral, se ha descrito que concentraciones incrementadas de IL-6 en plasma se asocian con un empeoramiento neurológico temprano (Vila et al., 2001), y niveles altos de ella predijeron deterioro neurológico temprano en el infarto cerebral (Castellanos et al., 2002), atribuyendo de esta manera un efecto dañino a esta IL. Por el contrario, otros hallazgos mostraron una correlación inversa significativa entre el nivel de IL-6 en plasma, daño neurológico y desarrollo de infarto cerebral, sugiriendo que su presencia en plasma puede representar efectos neuroprotectores en pacientes con lesión cerebral isquémica (Sotgiu et al., 2006). Por lo tanto, hay que tener en cuenta que cambios muy sutiles en los niveles de estos mediadores inflamatorios son difíciles de valorar e interpretar.

En la última década se ha progresado en el entendimiento del papel del sistema inmune y los procesos inflamatorios asociados a la patología del encéfalo. Con respecto a las fuentes de citoquinas en el SNC *in vivo*, se sabe que la mayoría de células no secretan citoquinas constitutivamente, sino que es la activación de la célula la que conlleva la transcripción del gen de la citoquina en concreto, desembocando en su secreción y liberación rápida (Tsuda, 2016). Por otro lado, una citoquina puede ser producida por muchos tipos celulares diferentes y tener múltiples efectos en diferentes tipos celulares también. No sólo las células gliales, sino también la microvasculatura del encéfalo afectado de EA son capaces de secretar grandes cantidades de citoquinas que a su vez pueden contribuir al daño neuronal (Zlokovic, 2011).

Por otro lado, el inicio de la acción de una citoquina se produce mediante su unión a receptores de superficie celular específicos en las correspondientes células diana. Dichos receptores muestran generalmente gran afinidad por sus ligandos, lo que implica que se necesita una muy pequeña cantidad de citoquina para que se inicie una respuesta biológica. La respuesta final de una célula diana está determinada por el nivel de expresión que esa célula presente, así como por las señales de transducción intracelulares que se activen cuando se produzca la unión ligando-receptor (Benveniste, 1992).

Se ha estudiado el papel específico que ejercen las citoquinas en las enfermedades neurodegenerativas humanas, concretamente en situaciones de neurodegeneración aguda tras lesiones o derrames cerebrales (Alan and Rothwell, 2001). También en la EA, en la que se sabe que aumenta la expresión de citoquinas (Dickson et al., 1993). Un estudio mostró interacciones entre citoquinas que influían en la muerte neuronal *in vitro*, usando cultivos celulares de encéfalos fetales humanos compuestos de neuronas y glía (Chao et al., 1995). De hecho, previamente Dickson et al., 1991 apuntaron que las células microgliales liberaban

citoquinas y quimioquinas en encéfalos afectados por todas esas enfermedades neurodegenerativas y accidentes cerebrovasculares.

También en patologías neurodegenerativas animales como el scrapie se ha demostrado que existe un perfil de citoquinas proinflamatorias alterado (Marcos-Carcavilla et al., 2007). Precisamente en esta enfermedad, se ha demostrado la inmunoreactividad y la inducción de las citoquinas IL-1β, IL-1α, IL-6 y TNF-α por células de la glía activada en encéfalos de ratones con síntomas clínicos (Liberski et al., 1990; Williams et al., 1994b; Campbell et al., 1994; Brown et al., 2003), aunque no sucede así en órganos periféricos tales como bazo, riñones o hígado (Campbell et al., 1994). La detección de la expresión de dichas citoquinas en encéfalo fue observada coincidiendo con el inicio de la sintomatología clínica, tanto en modelo murino infectado con scrapie como con ECJ (Campbell et al., 1994; Kordek et al., 1996). De hecho, Kordek et al., 1996, en un modelo experimental de ECJ en ratón encontraron una expresión 200 veces aumentada de TNF-α, IL-1α y GFAP, llegando a sugerir que la sobreexpresión de TNF-α durante el curso de la enfermedad puede mediar la vacuolización de la vaina de mielina observada en ECJ experimental. Sin embargo, hay que tener en cuenta que los niveles de expresión pueden variar dependiendo de la cepa murina utilizada y las diferentes combinaciones de inóculo de scrapie utilizados (Walsh, et al., 2001; Brown et al., 2003), por lo que los resultados de los modelos experimentales habría que interpretarlos con precaución y no serían totalmente fiables o extrapolables al curso real de la enfermedad.

En la enfermedad de scrapie se han llevado a cabo diversos estudios utilizando distintas técnicas para analizar los niveles de expresión de citoquinas, utilizando todos ellos modelos experimentales. Así, Williams et al., 1997b, estudiaron la presencia y localización de algunas de ellas mediante estudio inmunohistoquímico en encéfalos de ratones durante el curso de la infección con scrapie observando que los patrones de tinción de citoquinas eran más evidentes en tálamo, hipocampo y corteza cerebral que en el resto del encéfalo, como ya habían descrito en un estudio anterior (Williams et al., 1994b). Otros autores determinaron la expresión encefálica de IL-1β y TNF-α por PCR a tiempo real (RT-qPCR) en hámsters infectados (Servida et al., 2007). Más recientemente, en el trabajo de Tribouillard et al., 2009, se llevaron a cabo múltiples ensayos para analizar los niveles de 24 citoquinas en encéfalos de ratones infectados, mientras que en otros estudios utilizaron un kit comercial ELISA para detectar IL-1β, IL-6 y TNF-α en homogeneizados de encéfalos, observándose un aumento significativo de todas ellas (Xie, et al., 2013). Newsom et al., 2011 estudiaron una batería de citoquinas por *array* en ratones ovinizados en estadios preclínico y clínico. Todos los niveles de citoquinas

estaban elevados en todos los tejidos en fase preclínica, sugiriendo una respuesta compleja del hospedador antes de que los cambios histológicos y los signos clínicos aparezcan. En fase clínica, la respuesta de citoquinas fue dinámica y más notable en suero y tejidos periféricos que en el encéfalo.

Por todo ello, existen claras evidencias que hacen pensar que las citoquinas pueden ser factores significativos en el progreso de la patogénesis de la neurodegeneración, tanto en scrapie como en ECJ. De hecho, como ya se apuntaba con anterioridad en esta revisión, actualmente todas estas enfermedades neurodegenerativas se están estudiando desde el enfoque de la "teoría de la neuroinflamación", que en el caso concreto de las enfermedades priónicas se basa en el hecho de que existe una potente activación glial y como consecuencia, una gran producción de citoquinas *in vivo* (Williams et al., 1994b).

Sorprendentemente, estudios recientes han descrito un perfil de citoquinas tanto proinflamatorio como antiinflamatorio en un modelo murino infectado con cepas de scrapie clásico (Gómez-Nicola et al., 2013; Perry, 2016). Por lo tanto, existe un circuito de interacciones muy complejo mediado por citoquinas, por lo que su detección ha llegado a ser sumamente importante en el estudio de ésta, y para las enfermedades neurodegenerativas en general.

En base a la información extraída en relación con las citoquinas más relevantes en las respuestas inmunes e inflamatorias a partir de la extensa revisión bibliográfica realizada, en esta tesis doctoral nos centraremos en IL-1, IL-2, IL-6, IL-10, TNF-α e IFN-γ. Dependiendo de la localización y concentración de esas citoquinas en el SNC, habrá una inducción o bajada de la respuesta inmune. El resultado final de los procesos inmunológicos e inflamatorios que ocurran estará relacionado con las interacciones que sucedan entre estas citoquinas a nivel local. Adicionalmente, la caracterización de receptores de citoquinas ayudará a entender mejor la comunicación bidireccional que existe entre el sistema inmune y el sistema nervioso.

**i. IL-1**

Es producida predominantemente por macrófagos activados, aunque otros tipos celulares, incluyendo linfocitos B, células epiteliales, astrocitos y microglía, pueden secretarla cuando están activados (Arai KI et al., 1990). Se diferencian dos formas de esta citoquina, α y β, pero ambas se unen al mismo receptor de superficie y tienen actividades biológicas casi idénticas. Esta citoquina proinflamatoria es altamente inducible y activadora endógena de su receptor,

IL-1R. La secreción de IL-1 constituye el mayor estímulo para la activación de células T (que culmina con su rápida proliferación y expansión) mediante el aumento de expresión, tanto de IL-2 como de su receptor (IL-2R). Además, al igual que el TNF-α, la IL-1 estimula a varios tipos celulares a producir otras citoquinas como IL-6 o TNF-α o ella misma (Spulber et al., 2009).

La red neuronal en la que se activa el IL-1R junto con la activación diferencial de cascadas de señalización intracelular son determinantes del efecto de la IL-1 en la actividad neuronal (Vezzani et al., 2011) porque la expresión diferencial del receptor puede determinar si se activan programas de plasticidad o muerte de neuronas (Spulber et al., 2009). Por ejemplo, las quinasas MAP P38 y Src parecen estar predominantemente activadas por IL-1R1 en neuronas (Davis et al., 2006), mientras que es la vía NF-kB la que se activa cuando este receptor está activo en células gliales (Srinivasan et al., 2004). Es decir, la IL-1 podrá ejercer distintos efectos dependiendo de si se une a su receptor en un tipo celular u otro. Por otro lado, se ha observado que el mRNA del IL-1R se expresa en células de la microglía, astrocitos y neuronas de roedores, y también en células endoteliales de la BHE (Ban et al., 1991; Alheim and Bartfai, 1998). Además, se ha demostrado su implicación en enfermedades autoinmunes y enfermedades neurológicas crónicas (Dinarello, 2011).

Debido a este escenario tan complejo, la IL-1 es una de las citoquinas que más ampliamente se ha estudiado en el SNC (Alan and Rothwell, 2001). Se han llevado a cabo estudios que han mostrado que esta IL purificada resulta estimuladora para el crecimiento de astrocitos *in vitro* ( (Giulian and Lachman, 1985), mientras que si se inyecta directamente en el encéfalo puede estimular la astrogliosis (Giulian et al., 1988). Se ha sugerido que cuando es liberada por células inflamatorias puede contribuir a formar la cicatriz astroglial en encéfalos dañados de mamíferos (Benveniste, 1992). Además, induce cambios rápidos en la transmisión sináptica en el hipocampo (Viviani et al., 2007) y tiene la capacidad de reducir la liberación de neurotransmisores inhibiendo el voltaje de los canales dependientes de calcio (Viviani et al., 2007). En definitiva, estos estudios revelan que la IL-1 tiene efectos inhibitorios de la actividad neuronal.

Un estudio reciente demuestra que la microglía tiene potencial para autorenovarse y que la señalización del IL-1R participa en este proceso proliferativo y de restauración (Bruttger et al, 2015). Sin embargo, la microglía fagocítica se caracteriza precisamente por una falta de expresión de IL-1β (Hughes et al., 2010). Además, se ha descrito la presencia de astrocitos reactivos neurotóxicos inducidos por microglía activada y se ha determinado que este proceso se produce a través de la IL-1α y TNF-α (Liddelow et al., 2017).

En varias enfermedades neurodegenerativas, tanto priónicas como prion-*like*, se ha demostrado que la IL-1 puede estar sobreexpresada por células de la microglía (Mrak et al., 1995; Mark and Griffin, 2001; Brown et al., 2003). En condiciones patológicas (infección, lesión tisular o alteración inmunológica), dichas células gliales activan la expresión de algunas citoquinas (Sheng et al., 2011), entre las que, junto a TNF-α, figura la IL-1α (Fontana et al., 1982; Giulian et al., 1986; Hetier et al., 1990; Tchelingerian, et al., 1993).

En el scrapie, se ha descrito una sobreexpresión glial de esta IL en modelos murinos en estados preclínicos y clínicos de la enfermedad (Campbell et al., 1994; Brown et al., 2003). Algunos estudios han demostrado un perfil de citoquinas proinflamatorio alterado asociado con el tejido nervioso en el curso de la patología, aunque no sucede así en bazo u otros órganos periféricos (Campbell et al., 1994; Cunningham et al., 2005; Marcos-Carcavilla et al., 2007). En esta misma línea, otros autores han demostrado que los ratones deficientes en el gen que codifica la IL-1 son más resistentes al desarrollo del scrapie (Schultz et al., 2004). De hecho, Burwinkel et al., 2004 observaron que esta citoquina tenía una gran influencia en la patogénesis de dicha enfermedad en el modelo murino. Sus resultados sugieren que la IL-1 es un eje impulsor de la astrocitosis *in vivo*, aunque en el caso concreto del estadio terminal de scrapie, apuntan que, aparte de la IL-1, debe haber otros factores implicados, como podría ser la IL-6, cuya capacidad de activación de los astrocitos *in vitro* ha sido demostrada (Hafiz and Brown, 2000). Posteriormente, se sugirió que cualquier causa que conduzca a una desregulación de la actividad de la IL-1β podría influir en el inicio del estado clínico de la enfermedad de scrapie en ovino, corroborando que esta citoquina interfiere con el proceso neurodegenerativo (Marcos-Carcavilla et al., 2007).

#### ii. IL-2

Se trata de una citoquina secretada mayoritariamente por astrocitos (Eizenberg et al., 1995), que sirve como soporte trófico a las neuronas y a la glía (Awatsuji et al., 1993), aumentando el abrazamiento de las neuritas (Sarder et al., 1996), y que está implicada en el desarrollo dendrítico (Shen et al., 2010) y neuronal. CD25 constituye la cadena α de su receptor (IL-2R), y se expresa en células T y B activadas, así como en oligodendrocitos. Además, detecta células T reguladoras y efectoras (Baeyens et al., 2013).

Se han descrito efectos antiinflamatorios asociados a esta IL-2 (Saadoun et al., 2011), además de funciones inmunológicas pleiotrópicas (Klatzmann and Abbas, 2015). De hecho, a pesar de que su inmunoreactividad resultó profusa en tejido encefálico tanto de controles como de

pacientes de EA (Luber-Narod and Rogers, 1988), se ha descrito que este mediador puede mejorar la patología amiloide en ratones con esta patología (Alves et al., 2017).

En cuanto a las enfermedades priónicas, teniendo en cuenta toda la información revisada, no hay constancia de que se haya analizado el nivel de esta citoquina hasta el momento.

#### iii. IL-6

Este mediador, junto con las dos citoquinas mencionadas anteriormente, está implicado en la regulación de las respuestas inmunológicas e inflamatorias (Hirano and Kishimoto, 1989). Distintos tipos celulares, como los linfocitos B, linfocitos T, monocitos, células endoteliales, microglía y astrocitos secretan esta citoquina. Esta síntesis está a su vez inducida por otras sustancias, entre las que se incluyen IL-1, TNF-α e IFN-γ.

Se trata de la principal citoquina que induce la diferenciación de las células B activadas en células plasmáticas secretoras de inmunoglobulinas. En conjunto con su receptor soluble, IL-6R, juegan un papel importante en el proceso de transición desde la inflamación aguda a crónica mediante un cambio en la naturaleza del infiltrado leucocitario (de neutrófilos polimorfonucleares a monocitos/macrófagos) (Hurst et al., 2001; Gabay, 2006). Además, tanto IL-6 como IL-6R promueven la neurogénesis (Islam et al., 2009).

Se trata de una citoquina bastante peculiar, ya que, dependiendo del contexto, exhibe dos comportamientos opuestos. En modelos de inflamación aguda, exhibe un perfil antiinflamatorio (Xing et al., 1998), mientras que en modelos de inflamación crónica resulta ser proinflamatoria (Yamamoto et al., 2000).

Tiene un efecto mitogénico en los astrocitos (Selmaj et al., 1990), lo que podría contribuir a la gliosis reactiva. Al igual que para IL-1, hay dos fuentes endógenas de IL-6 en el SNC: los astrocitos y la microglía. Dado que la IL-6 es la citoquina que induce la diferenciación de células B en células plasmáticas secretoras de inmunoglobulinas, su producción por parte de astrocitos y microglía podría contribuir a intensificar las respuestas inmunes humorales detectadas en el SNC en algunas enfermedades neurológicas. En efecto, se ha demostrado que la IL-6 está elevada en el tejido del lóbulo temporal de pacientes afectados de EA (Wood et al., 1993), y que su inmunoreactividad se demuestra tanto dentro como alrededor de placas seniles en esta patología (Bauer et al., 1991; Strauss et al., 1992).

La expresión de esta IL fue aumentada por la rapamicina en astrocitos en un modelo murino de EP, lo que se asoció con una expresión reducida de citoquinas inflamatorias, poniendo de

manifiesto sus propiedades antiinflamatorias (Zhang et al., 2017). En resumen, puede actuar tanto como un factor proinflamatorio como antiinflamatorio (Tilg et al., 1997; Yasukawa et al., 2003).

Existen evidencias que demuestran las funciones tanto perjudiciales como protectoras de la IL-6 en el desarrollo de scrapie (Scherbel et al., 1999), a pesar de que algunos autores defienden que no tiene un papel fundamental (Mabbott et al., 2000). Toulmond et al., 1992 demostraron los efectos neuroprotectores de la IL-6 recombinante *in vivo.* Otros trabajos sugirieron que la IL-6 es importante para la neuroprotección, siendo crucial para la activación de las células gliales (Penkowa et al., 1999), pero también hay publicaciones en las que la sobre-expresión de IL-6 en encéfalos de ratones transgénicos resulta neurotóxica, provocando neurodegeneración (Campbell et al., 1993).

#### iv. IL-10

Es una citoquina antiinflamatoria e inmunomoduladora muy potente (Lee and Chau, 2002; Couper et al., 2008) que interfiere en la activación del eje NF-kB (una de las principales vías de señalización intracelular activada por citoquinas) (Planas et al., 2006) bloqueando la síntesis de otras citoquinas proinflamatorias (Wang et al., 1995).

Además, es un mediador importante de la intercomunicación entre microglía, astrocitos y neuronas (Lobo-Silva et al., 2016). Se ha descrito que esta citoquina es capaz de inhibir la producción de citoquinas proinflamatorias por la microglía, protegiendo así a los astrocitos de su activación excesiva (Ledeboer et al., 2002). Hay fármacos antiinflamatorios, como la atorvastatina, que inducen la producción de IL-10 *in vivo*, reduciendo el número de células microgliales activadas y en consecuencia, reduciendo también la producción de TNF-α (Ewen et al., 2013).

En términos generales, las citoquinas antiinflamatorias tienen propiedades neuroprotectoras (Hanisch, 2002). Así, Thackray et al., 2004 demostraron un desarrollo acelerado de las enfermedades priónicas en ausencia de esta IL, lo que hace pensar que de alguna manera es protectora frente a las enfermedades priónicas. Efectivamente, la deleción del gen que la codifica fue responsable de una extensión del tiempo de supervivencia en ratones (Thackray et al., 2004; Tamguney et al., 2008).

#### v. TNF-α

Se reconoce como un mediador de respuestas inflamatorias importante en un gran número de tejidos, funcionando como una citoquina inmuno-reguladora. Es secretado mayoritariamente

por macrófagos activados, y en menor medida por otras células, como linfocitos T, astrocitos y microglía (Chu, 2017). Además, estimula a otros tipos celulares para producir citoquinas como IL-1 o IL-6, o ella misma. También puede regular las respuestas inmunes modulando la expresión de moléculas del MHC I y II en varios tipos celulares, incluidos los astrocitos y la microglía.

En el SNC, al igual que para IL-1 e IL-6, existen dos fuentes endógenas de TNF-α: astrocitos y microglía. Éste se produce en respuesta a la exposición de las células gliales a IFN-γ e IL-1 (Bethea et al., 1992). En concreto, la microglía en modelo murino lo secreta en respuesta a IFN-γ (Frei et al., 1987). El hecho de que los astrocitos puedan secretar esta citoquina, y ésta inducir la consecuente producción de IL-6 por parte de los mismos astrocitos, puede considerarse como una vía reguladora negativa para controlar la expresión de TNF-α en el SNC.

Se ha descrito que el TNF-α liberado por la microglía está aumentado en todas las enfermedades neurodegenerativas (Carvey et al., 2009). Así, una de las vías por las que la microglía puede ejercer daño o muerte neuronal es mediante la liberación de este mediador (Brown and Vilalta, 2015; Hickman et al., 2018). Tiene un gran rango de funciones en el SNC por su influencia en astrocitos (los activa para que secreten algunas citoquinas, entre ellas la IL-6) (Benveniste et al., 1990). De hecho, la astrogliosis reactiva se asocia con la liberación de este factor (Wang et al., 2015). El TNF-α es uno de los diferentes estímulos capaz de desencadenar la liberación de glutamato desde el astrocito (Rossi et al., 2005). Si se co-cultiva microglía reactiva con astrocitos, imitando lo que ocurre durante una inflamación glial aguda, los niveles de TNF-α aumentan de forma muy significativa. Esto causa una fuerte amplificación de la liberación de glutamato dependiente del TNF-α, que hasta cierto punto puede inducir muerte neuronal apoptótica (Bezzi, et al., 2001). Por el contrario, también se han descrito propiedades neuroprotectoras de dicho mediador (Bruce et al., 1996; Scherbel et al., 1999). Por lo tanto, es un hecho destacable el que se hayan atribuido propiedades tanto neurotóxicas como neuroprotectoras a este mediador.

La sobrerregulación de su correspondiente receptor, TNFR, se ha descrito en enfermedades neurodegenerativas como EP (Scalzo et al., 2009), así como en cerebelos de ratones infectados con scrapie (Ragagnin et al., 2017). En encéfalos de EA se han descrito niveles elevados, tanto de TNF-α como de su receptor TNFR-1 (Zhao et al., 2003), que se expresa tanto en neuronas como en células gliales (Dopp et al., 1997). Además, en cultivos celulares, los péptidos de β-amiloide causan la liberación del TNF-α por parte de la microglía.

En las enfermedades priónicas, este mediador está implicado en la diferenciación y maduración de las CDF, necesarias para que haya acúmulo de $PrP^{sc}$ en el bazo y se produzca posteriormente la neuroinvasión (Brown et al., 1999; Mabbott et al., 2000). De hecho, se ha descrito que en animales *knockout* esta proteína es responsable de un incremento en el periodo de incubación del scrapie (Mabbott et al., 2002). También se ha demostrado que el TNF-α está implicado en la patogénesis de la ECJ tipo panencefálico (Liberski et al., 1995), participando en la degradación de la mielina de los axones. Todo ello se ha relacionado con una aceleración del proceso neurodegenerativo en respuesta a esta citoquina inducida por la acumulación del prion. Tras la reciente descripción de los astrocitos reactivos neurotóxicos, inducidos por la microglía activada a través de las citoquinas IL-1α y TNF-α (Liddelow et al., 2017), el TNF-α estaría contribuyendo a la comunicación entre microglía y astrocitos, y por ende, al desarrollo de la neurotoxicidad priónica.

**vi. IFN-γ**

Es un producto de las células T activadas y está presente en el SNC solamente en las situaciones patológicas en las que se ha roto la BHE y las células T han infiltrado el encéfalo. Es una citoquina con actividad antiviral, efecto antiproliferativo e inmunomodulador que promueve la diferenciación tanto de células T como B (Binder and Griffin, 2001; Rottenberg and Kristensson, 2002). El complejo receptor de esta citoquina está formado por dos subunidades, el IFN-γR1 e IFN-γR2 (Bach et al., 1997), siendo necesarias ambas para una actividad biológica completa.

Fisiológicamente, tanto astrocitos como microglía expresan niveles muy bajos de antígenos del MHC I y no expresan antígenos del MHC II. Pero IFN-γ puede inducir un incremento en la expresión de MHC I en astrocitos, oligodendrocitos y microglía (Hirayama et al., 1986), y también de MHC II (Suzumura et al., 1987). En general, se considera que es el inductor más potente de expresión de MHC II en la mayoría de tipos celulares, excepto en células B (Cogswell et al., 1991). En cuanto a su funcionalidad, Vass and Lassman, 1990, propusieron que la microglía que expresa MHC II tiene un papel importante en el inicio de la presentación de antígenos en el SNC, mientras que los astrocitos, pueden estar implicados en la propagación de reacciones inmunes en el SNC. Así, puede actuar para suprimir, en última instancia, las respuestas inmunes e inflamatorias, mediante la inhibición de la expresión de MHC I y II y de la producción de citoquinas por parte de las células gliales (Benveniste et al., 1992).

Además, el TNF-α aumenta la expresión de MHC II inducido por IFN-γ en astrocitos (Vidovic et al., 1990). Por otro lado, IFN-γ induce un incremento númerico de la expresión de receptores

para TNF-α en astrocitos de rata (Benveniste et al., 1989), lo que puede contribuir a la sinergia para mediar efectos biológicos ya descrita entre estas dos citoquinas. De todas formas, el IFN-γ no parece inducir directamente la producción de citoquinas por parte de los astrocitos, sino que constituye una "señal de preparación" para que el astrocito responda tras la exposición a otras citoquinas. Por ejemplo, los cultivos de astrocitos pre-tratados con IFN-γ y posteriormente expuestos a IL-1, producen más TNF-α en comparación con el efecto que tiene la adición simultánea de ambas citoquinas (Chung and Benveniste, 1990). Esto sugiere que el IFN-γ genera una señal de preparación en el astrocito que después incrementa su sensibilidad a la exposición de IL-1, enfatizando de nuevo la complejidad de las interacciones entre citoquinas, y demostrando que las distintas respuestas dependen incluso de la secuencia temporal de liberación de estos mediadores.

Cuando la microglía residente invade una zona lesionada del SNC, secreta citoquinas que estimulan su división, entre las cuales se encuentra el IFN-γ y también IL-1β e IL-4 (Kim and de Vellis, 2005). También se ha demostrado que la microglía regula la neurogénesis adulta de manera específica, tanto en el espacio como en el tiempo, y que tales efectos neurogénicos están mediados por una combinación de citoquinas, entre las que se encuentra dicho IFN-γ (Sato, 2015).

Todos estos mediadores pro y antiinflamatorios contribuyen de manera sustancial al desarrollo de la neuroinflamación en todas las enfermedades neurodegenerativas.

### *c.* Neuroinflamación en las enfermedades neurodegenerativas

Hace unos 25 años que se describió por primera vez la existencia de una respuesta inflamatoria innata en la EA (Akiyama, 1994). Dos hechos básicos condujeron a que se empezara a emplear el término "neuroinflamación"; por un lado, la demostración mediante IHQ de microglía reactiva en EA, que confirmó la existencia de una inflamación crónica en esos encéfalos (McGeer et al., 1987; Luber-Narod and Rogers, 1988); por otro, personas que sufrían artritis reumatoide tenían un riesgo reducido de padecer EA debido al largo tratamiento con fármacos antiinflamatorios no esteroideos (AINEs) que habían recibido (McGeer et al., 1990). Por lo tanto, la primera vez que se describe este término como tal fue en los años 90 para la EA.

Posteriores estudios han ido descubriendo componentes inflamatorios en la EP, ELA, EM y enfermedades priónicas (Glass et al., 2010; Carroll and Chesebro, 2019). En la actualidad,

existe un particular interés en investigar el papel de los sistemas inmunes innato y adaptativo en todas estas enfermedades neurodegenerativas (Calsolaro and Edison, 2016; Carroll and Chesebro, 2019).

La visión de la microglía como la célula conductora de la respuesta neuroinflamatoria con consecuencias neuropatológicas condujo al concepto moderno de "neuroinflamación" (Streit et al., 2004). La neuroinflamación, definida como la activación de la microglía sostenida en el tiempo, con la consecuente producción de citoquinas proinflamatorias y especies reactivas de oxígeno, es una característica propia de diferentes neuropatologías (Schwartz and Deczkowska, 2016). Así, las características que la definen son: el aumento de citoquinas proinflamatorias, la activación microglial y la neurodegeneración secundaria (Estes and McAllister, 2014; Kempuraj et al., 2016; Carroll and Chesebro, 2019). Por lo tanto, la respuesta inflamatoria central representa una reacción inflamatoria crónica localmente inducida y atípica. En las enfermedades priónicas existe un patrón de neuroinflamación poco frecuente, manifestado no sólo por los infiltrados celulares atípicos sino también por los perfiles de expresión génica de citoquinas inusuales (Perry et al., 2002), probablemente debido a la existencia de diferentes cepas priónicas, lo cual debería tratarse como si fueran distintas enfermedades.

Sin embargo, a día de hoy se conoce que no sólo es la microglía, sino también los astrocitos los que tienen un papel activo en la inmunidad cerebral innata (Farina et al., 2007), habiéndose demostrado previamente por parte de nuestro grupo de investigación que están altamente implicados tanto en scrapie (Sarasa et al., 2012; Hernández et al., 2014) como en enfermedades priónicas humanas (Monzón et al., 2018) y prion-*like* (Garcés et al., 2019), poniendo de manifiesto que el modelo natural de scrapie puede ser un modelo fiable para extrapolar a otras enfermedades neurodegenerativas. Así, actualmente se considera que la neuroinflamación que está asociada con la infección priónica se caracteriza por la activación de ambas poblaciones gliales, astrocitos y microglía (Aguzzi et al., 2013; Carroll and Chesebro, 2019), proceso que culmina con la neurodegeneración (Ransohoff, 2016). Dichas respuestas inflamatorias sostenidas en el tiempo contribuyen a la progresión de la enfermedad a través de las citoquinas proinflamatorias que liberan; por lo tanto, se ha especulado que la combinación de factores liberados por microglía activada y los astrocitos puede promover la neurotoxicidad (Morales et al., 2014; Serpente et al., 2014; Lyman et al., 2014), como se ilustra en la Figura 2.

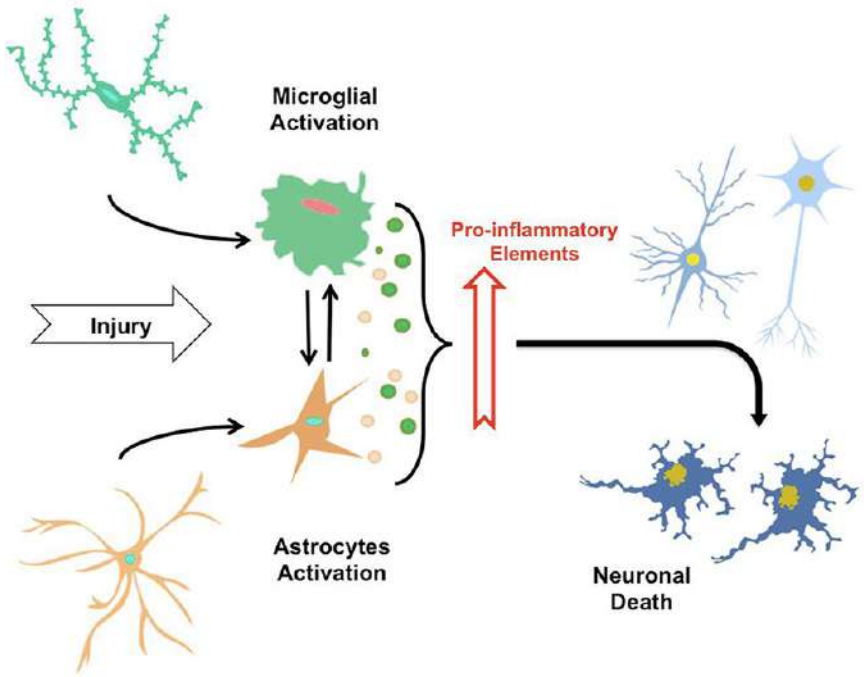


**Figura 2.** Esquema en el que se ilustra cómo la neuroinflamación puede promover la neurotoxicidad (Morales et al., 2014).

Sin embargo, el concepto emergente de la inducción de funciones no sólo protectoras, sino también tóxicas, de la microglía (Falsig et al., 2008; Xie et al., 2013; Carroll et al., 2016; Zhu et al., 2016; Iaccarino et al., 2018) y los astrocitos (Riemer et al., 2009; Liddelow et al., 2017), hace que actualmente se hayan descrito aspectos tanto positivos como negativos de la neuroinflamación, ya que la intensidad y duración de la respuesta inflamatoria podría determinar si las señales inmunológicas resultan finalmente beneficiosas o destructivas para el SNC (DiSabato et al., 2016), como se resume en la Figura 3.

Con respecto a los aspectos positivos, la respuesta inflamatoria controlada generalmente se considera beneficiosa para el organismo; son los casos de las infecciones, el aprendizaje y la memoria, la remodelación de lesiones traumáticas del SNC o situaciones de euflamación. Por ejemplo, las señales inmunes que se producen tras las infecciones conducen a la reorganización de las prioridades del hospedador, ya que una respuesta coordinada mediada por la unidad neurovascular y la glía residente propaga las citoquinas y las señales secundarias que causan los componentes fisiológicos y de comportamiento de la enfermedad, incluyendo la fiebre, entre ellos (Berg et al., 2004; Dantzer et al., 2008). Además, hay un mantenimiento importante del papel de la IL-1 e IL-4 en el aprendizaje y la memoria (Ziv et al., 2006; Derecki et al., 2010). Tras la lesión traumática del SNC, la repolarización de la microglía a fenotipo macrofágico M2 producida por IL-4 ha resultado ser altamente efectiva promoviendo la

recuperación y el crecimiento axonal de nuevo (Gensel et al., 2009; Kigerl et al., 2009). Por último, el condicionamiento immune o euflamación (activación temporal del sistema inmune previo a una lesión o infección) se considera un método para entrenar el sistema immune hacia un fenotipo más neuroprotector (Tarr et al., 2014).

En cuanto a los aspectos negativos de la neuroinflamación, destacan las respuestas inflamatorias inadecuadas (a causa de lesiones traumáticas del SNC, estrés social, edad o enfermedades neurodegenerativas). Por ejemplo, la inflamación crónica incontrolada se caracteriza por una producción incrementada de citoquinas (IL-1 y TNF-α), especies reactivas de oxígeno y otros mediadores inflamatorios como la enzima inducible óxido nítrico sintetasa. Todos estos marcadores muy evidentes tras un trauma en el SNC (Shultz et al., 2013; Huang et al., 2014). Las especies reactivas de oxígeno están acompañadas del reclutamiento y tráfico de macrófagos periféricos y neutrófilos al sitio lesionado (Semple et al., 2010). El estrés social también conlleva una inflamación temporal que conduce al reclutamiento de monocitos y macrófagos y causa ansiedad y depresión (Wholeb et al., 2014). Además, con la edad, va surgiendo una respuesta inflamatoria crónica a bajo nivel en la que hay IL-1 e IL-6 y conduce a una plasticidad neuronal reducida y deficiencia cognitiva (Xie et al., 2003; Godbout et al., 2005; Sierra et al., 2007; Chung et al., 2009). Por último, las enfermedades neurodegenerativas producen un gran nivel de inflamación crónica que es altamente dañina para el SNC (Mrak et al., 1995; Sokolova et al., 2009).

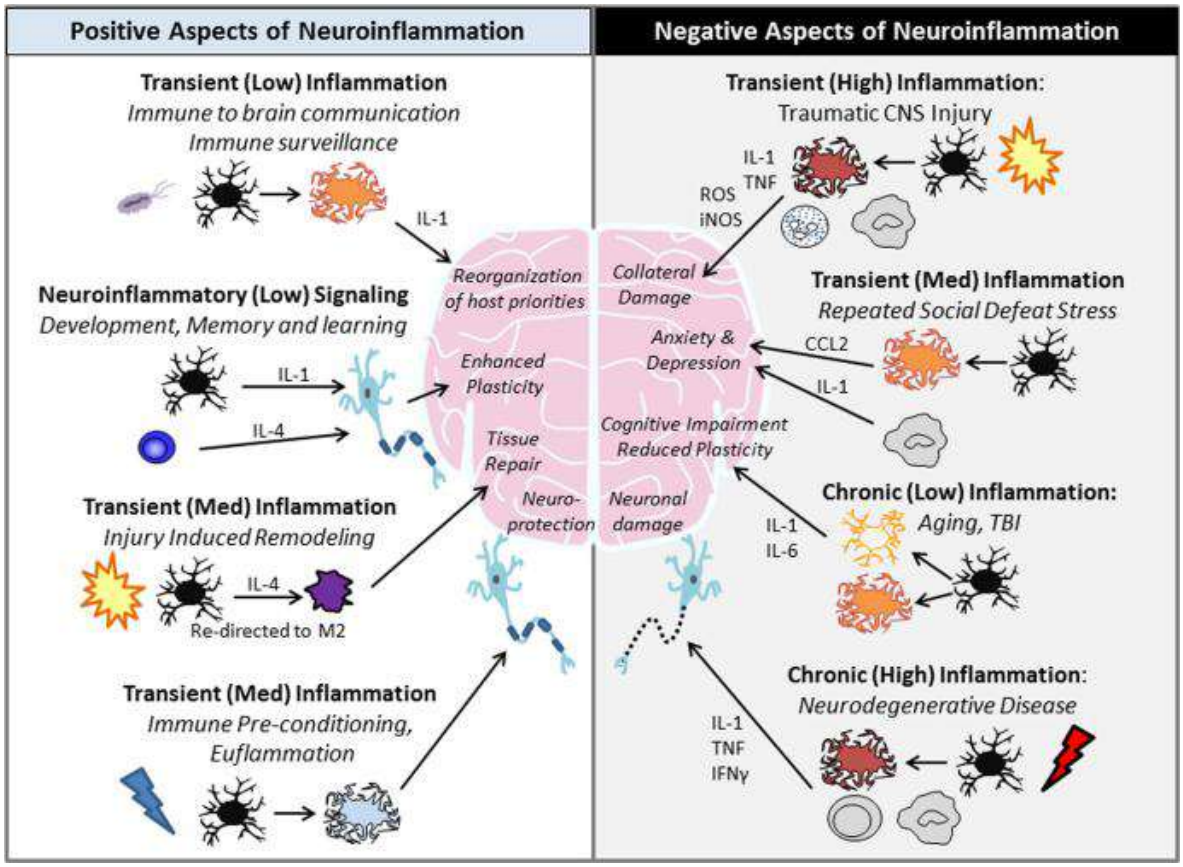


**Figura 3.** Aspectos beneficiosos y perjudiciales de la neuroinflamación (DiSabato et al., 2016).

Es difícil extraer conclusiones generalizando sobre la positividad o negatividad de la neuroinflamación, ya que debido a la complejidad del proceso quizás la mejor forma de realizar un resumen es que se necesita estudiar cada caso en profundidad dependiendo del contexto.

A pesar de todo, es necesario tener en cuenta que no todas las alteraciones en expresión de mediadores o células inmunes conllevan necesariamente asociado un proceso de neuroinflamación, ya que algunos mediadores son patológicos en un contexto de enfermedad pero pueden ser terapéuticos en otra (Estes and McAllister, 2014). Es el ejemplo del TNF-α en la esclerosis múltiple, ya que una respuesta inmune típicamente asociada con inflamación puede funcionar paradójicamente para atenuar la patogénesis de una enfermedad neurodegenerativa (Balosso et al., 2005).

Teniendo en cuenta todo lo descrito sobre neuroinflamación, aunque actualmente no exista un tratamiento para las enfermedades priónicas, muchos han sido los grupos de investigación que durante las últimas décadas han intentado y siguen intentando abordar el problema mediante distintas estrategias terapéuticas, principalmente centradas en dichos aspectos inflamatorios del SNC.

## 4. TERAPIAS UTILIZADAS EN LAS ENFERMEDADES PRIÓNICAS

No sólo fue la pasada epidemia de EEB la responsable de que se empezaran a buscar tratamientos para las enfermedades priónicas, sino la epidemia actual de enfermedad caquectizante crónica del ciervo, que podría transmitirse a los humanos, lo que hace que exista una necesidad urgente de terapias efectivas. Muchos han sido los grupos de investigación que han estudiado diversas opciones de tratamientos para prevenir la propagación del prion.

En cuanto a terapias para enfermedades humanas, los esfuerzos realizados en busca de nuevos tratamientos frente a enfermedades neurodegenerativas se han realizado principalmente utilizando modelos de patologías prion-*like*. Entre ellos, se ha valorado la posible eficacia de los fármacos antiinflamatorios más ampliamente usados desde hace décadas, que son los AINEs (Van Muiswinkel and Eikelenboom, 1996). En concreto, existen algunos estudios epidemiológicos que mostraron que su uso podía retrasar la edad de inicio de la EA (Lucca et al., 1994; McGeer et al., 1996). Otros estudios sugirieron una posible neuroprotección utilizando estos fármacos, al relacionarse con un riesgo reducido de padecer EP (Gagne and Power, 2010). Los tratamientos con ibuprofeno y naproxeno como principales AINEs administrados en la actualidad mostraron una reducción significativa en la activación microglial (Yan et al., 2003; Varvel et al., 2009). Sin embargo, cuando ya se ha iniciado el depósito de β-amiloide, no existe efecto del AINE y puede ser incluso dañino, al inhibir la actividad microglial, que ayuda a fagocitar la proteína y activa la neurogénesis hipocampal compensatoria (Imbimbo, 2009).

Más tarde, se sugirió que los glucocorticoides (GC) y no los AINEs, estaban asociados con una más leve neuropatología en EA que resultaba en beneficios clínicos (Beeri et al., 2012). Además, se demostró que el GC dexametasona (DEX), usada a una dosis correcta, protegía frente al daño de las neuronas dopaminérgicas que se produce en la EP (Kurkowska-Zabreska et al., 2004). Años después, Tentillier et al., 2016 volvieron a conseguir neuroprotección

dopaminérgica modulando la inflamación mediante GC en la EP, por lo que se apoyaba el uso de antiinflamatorios para el tratamiento de la neuroinflamación crónica.

Otros estudios han incluido el tratamiento de ratones con linfotoxina LTβR-Ig (factor liberado por linfocitos activados que está implicado en la apoptosis y liberación de citoquinas). Su administración de forma previa a la exposición de una dosis relativamente baja de priones causantes de ECJ seguido por otras dos semanas de tratamiento post-inoculación, tuvo como resultado la protección completa de esos ratones frente a la enfermedad (Aguzzi and Heikenwalder, 2005).

Anle138b, que presenta la capacidad de inhibir la agregación proteica, es un modulador de oligómeros bastante novedoso que se ha usado en estudios recientes para probar su eficiencia en patologías neurodegenerativas. Éste ha sido seleccionado entre otros 150 componentes relacionados con el di-fenil-pirazol debido a su eficacia terapéutica *in vivo*. El tratamiento oral de ratones iniciado 120 días post-inoculación y tras el inicio de la enfermedad, prolongó el mayor tiempo de supervivencia de entre todos los componentes farmacológicos probados en tratamientos experimentales a largo plazo (Wagner et al., 2013).

Además de terapias en humanos, para prevenir el riesgo zoonótico de las enfermedades priónicas, se han intentado buscar estrategias terapéuticas en animales, principalmente en scrapie. Sin embargo, la mayoría de los estudios se han realizado en modelos experimentales.

El primer estudio en el que se probó un fármaco frente al prion *in vivo* fue en 1974. Outram et al., 1974 trataron ratones con esteroides y observaron que se reducía la susceptibilidad al scrapie. Dicho tratamiento prolongó el periodo de incubación (con un inicio de enfermedad clínica siete meses después) y tuvo como resultado un 20% de supervivientes a largo plazo (individuos que estaban vivos y sanos 200 días después de la muerte de todos los ratones que no recibieron tratamiento esteroideo).

Poco después, en 1979, se inocularon ratones con scrapie tanto por vía intraperitoneal como subcutánea y se demostró un efecto supresor del HPA-23 (sal derivada del amonio utilizado como antiviral) (Kimberlin and Walker, 1979). Cuatro años después, se amplió dicho estudio utilizando tres modelos animales experimentales diferentes e inoculados con diversas cepas priónicas (Kimberlin and Walker, 1983). El tratamiento aplicado durante 13 ó 28 días post-inoculación retrasó los periodos de incubación significativamente, aunque el efecto era mucho mayor cuando se aplicaba el tratamiento en el mismo momento de la inoculación. En base a

estos resultados, se propuso que la administración parenteral de dosis altas de este fármaco podría ser útil como terapia profiláctica de enfermedades priónicas humanas iatrogénicas.

La anfotericina B es un antifúngico y antibiótico que se ha estudiado por su eficiencia frente al scrapie en diferentes modelos en roedores. Sin embargo, tras varios diseños con diferentes líneas murinas y cepas priónicas, evidenció que este compuesto sólo era terapéuticamente efectivo frente a alguna cepa priónica (Demaimay et al., 1994).

Algunas otras estrategias terapéuticas se han centrado de forma específica en la acción de sustancias con propiedades antiinflamatorias, como es el caso del cannabidiol (sustancia no psicoactiva que se une a los receptores cannabinoides del organismo y provoca efectos parecidos a la planta *Cannabis sativa*), que fue capaz de aumentar el tiempo de supervivencia en ratón y prevenir el acúmulo de $PrP^{sc}$ en cultivos celulares infectados con scrapie (Dirikoc et al., 2007). La simvastatina, un regulador del colesterol con propiedades antiinflamatorias, retrasó el avance del scrapie en ratón (Haviv et al., 2008). De Luigi et al., 2008 mostraron los efectos beneficiosos de las tetraciclinas administradas tanto por vía intramuscular como por vía subcutánea en hámsters infectados con la cepa priónica 263 K. La doxiciclina retrasó la enfermedad en hámsters infectados con scrapie, aunque cuando se administró a pacientes, este fármaco no tuvo efecto significativo (Haik et al., 2014). Más recientemente, Shah et al., 2017 demostraron que el tratamiento temprano (en fase preclínica) con minociclina mejoraba la supervivencia y aliviaba la neuroinflamación y neurodegeneración en hámsters infectados con scrapie.

También el guanabenz (un fármaco agonista adrenérgico α2 utilizado normalmente para la hipertensión), con capacidad antiinflamatoria demostrada, fue activo *in vivo* en el modelo de scrapie murino Tg338 (Tribouillard-Tanvier et al., 2008).

Teniendo en cuenta todos los estudios anteriores, resulta evidente la variabilidad de resultados en función de la combinación del modelo animal y la cepa priónica, incluida la capacidad de replicación previamente a la neuroinvasión, al evaluar el efecto de cualquier fármaco.

Por otro lado, varios autores han sugerido que la estimulación de vías de neuroprotección que retrasen el inicio de la enfermedad en el SNC podrían representar una posibilidad terapéutica. Por ejemplo, Burwinkel et al., 2004 demostraron que una deficiencia en la interacción de la vía CD40 (un miembro de la superfamilia de los receptores del TNF-α) con su ligando conduce a un

desarrollo rápido de la enfermedad de scrapie en ratones infectados comparados con los ratones *wild type* o control, que no presentaban esta deficiencia.

Ya se ha descrito previamente el papel de la apoptosis como mecanismo en la neurodegeneración asociada a enfermedad priónica (Kretzschmar et al., 1997; Williams et al., 1997a), por lo que también se ha postulado que se podrían tomar como diana terapéutica las cascadas de señalización proapoptóticas o utilizar la sobre-expresión de proteínas antiapoptóticas (Burwinkel et al., 2004).

En esta misma línea, Xu, et al., 2015 probaron varios fármacos en cultivos celulares de microglía y demostraron que uno de ellos tenía actividad antiinflamatoria potente, ya que inhibía la activación de la microglía. Incluso sugirieron que podría servir como herramienta terapéutica para el tratamiento de enfermedades neuroinflamatorias, ya que dicho fármaco suprimió la expresión de TNF-α, IL-6, y COX-2, además de la enzima óxido nítrico sintetasa.

Hasta ahora, se mantenía el dogma de que cualquier estrategia terapéutica debería tener como objetivo prevenir la actividad microglial excesiva a la vez que retener sus funciones protectoras. No obstante, debido a la reciente descripción de astrocitos reactivos neurotóxicos que son inducidos por microglía activada a través de las citoquinas IL-1α y TNF-α (Liddelow et al., 2017), actualmente se deberían buscar fármacos que prevengan la formación de este tipo astrocítico o que bloqueen la neurotoxina liberada por ellos para poder encontrar finalmente un tratamiento efectivo frente a las enfermedades neurodegenerativas crónicas.

Aunque existen muchas líneas de investigación prometedoras que buscan terapias efectivas, lamentablemente, a día de hoy, sigue sin haber un tratamiento efectivo frente a ellas. Al tratarse de enfermedades degenerativas crónicas, es posible que su prevención y tratamiento requerirá una terapia a largo plazo, con fármacos que tengan un alto nivel de seguridad (Mallucci and Collinge, 2005). El riesgo más obvio es la supresión de la función inmune innata hasta el punto de permitir el desarrollo de infecciones oportunistas.

Cuarenta años después de que se probara el primer fármaco frente al prion en un modelo animal, aún se continúa con la búsqueda de terapias que puedan ser extrapolables a humanos. La existencia de cepas priónicas hace que las enfermedades priónicas se presenten como diversas entidades patológicas con diferentes signos clínicos, perfiles lesionales y patogenicidad. Es por ello por lo que la búsqueda de un componente frente al prion que sea efectivo para todas las enfermedades priónicas resulta francamente complicado. Por ello, actualmente los esfuerzos se han redirigido a la utilización de modelos animales fiables para

poder extrapolar resultados y llegar algún día a mitigar entre otras, la enfermedad priónica humana más común, la eECJ (Fernández-Borges et al., 2013).

Debido a la naturaleza compleja de estas enfermedades y al número de tipos celulares implicados, puede que se necesite la potencial sinergia de más de un fármaco para obtener un beneficio terapéutico, como se sugirió en otras enfermedades como las sinucleinopatías (Valera and Masliah, 2016).

Teniendo en cuenta la falta de éxito en la búsqueda de terapias frente este grupo de enfermedades, unido a que todos los estudios terapéuticos se han llevado a cabo utilizando modelos experimentales, en esta tesis doctoral nos hemos centrado en valorar el efecto que los glucocorticoides tienen sobre la neuroinflamación, utilizando para ello el modelo natural de scrapie ovino. Por ello, en el capítulo final de esta introducción se incluye una revisión bibliográfica sobre el papel de estas hormonas en el SNC y en concreto, en la patología neurodegenerativa.

## 5. GLUCOCORTICOIDES

Los GC son hormonas liberadas durante la respuesta de estrés que son bien conocidas por sus propiedades inmunosupresoras (Dhabhar and McEwen, 1997) y antiinflamatorias. La primera descripción de que la respuesta de estrés podía tener efecto en la inmunidad la realizó Selye en 1936, cuando observó que el estrés crónico iba asociado con la atrofia del timo (Szabo et al., 2017).

### a. Glucocorticoides como hormonas endógenas. Respuesta de estrés

Las hormonas esteroides son una familia de mediadores derivados del precursor común colesterol que están implicadas en las respuestas de estrés e incluyen como grupo principal a los glucocorticoides. Se ha descrito que dichas hormonas tienen propiedades neuroprotectoras (Garcia-Ovejero et al., 2005).

En humanos, ovejas y monos, el cortisol es la principal hormona glucocorticoide. Sin embargo, la corticosterona (17-deoxicortisol) es la principal hormona adrenal secretada en pájaros, ratones, ratas y conejos (que son los animales principalmente utilizados para experimentos de actividad de los fármacos glucocorticoides) (Sorrells et al., 2009). Ambas hormonas regulan el metabolismo de lípidos, proteínas e hidratos de carbono, controlan las respuestas de control al distrés y enfermedad y ayudan a mantener la homeostasis del sistema inmune y esqueleto.

Tanto el cortisol como la corticosterona se sintetizan en función de la demanda del organismo y pasan inmediatamente a la circulación general (sangre y linfa). Su síntesis en la glándula adrenal (GA) se controla mediante el eje hipotálamo-hipófisis-adrenal (HPA), que resulta clave para inhibir la regulación de la respuesta inflamatoria (Morand and Leech, 2001).

Los efectos de los GC endógenos están mediados por dos tipos de receptores intracelulares que están relacionados, el receptor tipo I o receptor de mineralocorticoides, y el receptor tipo II o receptor de glucocorticoides (GR) (de Kloet et al., 1993). Los GR están presentes en las neuronas del hipocampo (Van Steensel et al., 1996) y tienen alta afinidad por el GC sintético DEX, aunque no así por la corticosterona (Veldhuis et al., 1982). Ambos tipos de receptores se encuentran en regiones encefálicas discretas, especialmente en el sistema límbico, y ejercen control inhibitorio sobre el eje HPA; además, afectan a la respuesta de neurotransmisores. De hecho, está descrito que los GR microgliales pueden jugar un papel en la regulación de la neurodegeneración de la EP (Ros-Bernal et al., 2011).

Miller et al., 1992 examinaron la relación entre la dosis de DEX y la respuesta de activación de GR en la hipófisis y el encéfalo. Encontraron que bajas dosis de DEX producían activación selectiva de GR en la hipófisis, mientras que en el encéfalo no se veían afectadas. Sin embargo, con altas dosis de DEX sí se conseguía activar los GR en encéfalo. Por lo tanto, los GC son hormonas esteroides que rápidamente cruzan la BHE, se unen a GR con baja afinidad y a receptores de mineralocorticoides con alta (Reul and de Kloet, 1985).

Actualmente, existe un interés creciente en cómo estas hormonas de estrés afectan a la inflamación, con un enfoque particular al SNC. Como punto clave, la capacidad antiinflamatoria a veces falla, e incluso, puede empeorar la inflamación en el SNC dañado. La paradoja es que niveles basales de GC son esenciales para el desarrollo neuronal, plasticidad y supervivencia, mientras que niveles de estrés de GC producen pérdida neuronal (Reagan and McEwen, 1997). Dicha paradoja es muy evidente en el hipocampo, la región encefálica que sirve como centro de integración crítico para la memoria (McEwen and Sapolsky, 1995). La apoptosis representa la vía común final de pérdida neuronal en el hipocampo en respuesta a una variedad de procesos mediados por GC.

De forma fisiológica, la respuesta de estrés comienza cuando el encéfalo detecta un cambio homeostático, activa el sistema nervioso simpático, que libera catecolaminas (epinefrina y norepinefrina). Seguidamente, se activa el eje HPA, que provoca que el hipotálamo libere corticotropina (CRH) y la hipófisis secrete la hormona adenocorticotropa (ACTH), que acaba estimulando la secreción de GC en la zona fasciculata de la GA (Webster et al., 2002; Howell

and Muglia, 2006). Es necesario destacar que el funcionamiento de este eje HPA muestra una tremenda variabilidad individual, hecho que ayuda a explicar las diferencias entre individuos en cuanto a su vulnerabilidad a la enfermedad relacionada con el estrés.

El eje HPA es regulador de la respuesta inmune y es un mecanismo clave para la regulación inhibitoria de la respuesta inflamatoria (Morand and Leech, 2001). Las hormonas son inmunomoduladores potentes de cada paso del proceso inflamatorio, y, por lo tanto, las citoquinas inflamatorias modulan el eje HPA (Foster et al., 2003). Sin embargo, una secreción sostenida de cortisol podría exacerbar el proceso neurodegenerativo lento que sucede en las enfermedades priónicas. En efecto, se ha demostrado que la exposición excesiva a GC tiene efectos nocivos en el encéfalo de roedores (Sapolsky, 1996).

Debido a los efectos *in vivo* de los GC endógenos producidos por las glándulas adrenales, surgieron versiones de GC sintéticos con efecto antiinflamatorio.

**b. Glucocorticoides sintéticos**

Muchos textos médicos y revisiones describen el descubrimiento y desarrollo de los GC como fármacos antiinflamatorios, originarios de la hormona endógena cortisona (Hillier, 2007), que estuvo disponible por primera vez hace 60 años (Whitehouse, 2011). Dichos GC se han usado durante casi 70 años para el tratamiento de varias enfermedades con componente inflamatorio, como la artritis reumatoide (Kirwan et al., 1999) y enfermedades autoinmunes (Rosen and Miner, 2005). Están entre los fármacos más universalmente prescritos, basándose en la reducción del proceso inflamatorio y en su potencia inmunosupresora (Devogelaer, 2006).

En cuanto a su modo de acción, se conoce que son potentes inhibidores de la vía NF-kB (Auphan et al., 1995; Grilli and Memo, 1999). La familia NF-kB activa la expresión de factores de transcripción que participan en la activación de un amplio rango de genes crucialmente implicados en funciones inmunes e inflamatorias o de forma más general, en la respuesta al estrés. Si esta activación es parte del proceso neurodegenerativo o de mecanismos neuroprotectores es aún objeto de debate. Se inicia por señales inflamatorias (como el TNF-α) (Schütze et al., 1992) y a su vez activa genes implicados en la inflamación (Grilli and Memo, 1997).

De forma sintética, se han postulado diversas versiones de GC. No son simples imitaciones del cortisol. Por ejemplo, algunas características que los diferencian de la hormona endógena son: afectan al sistema inmune intestinal tras la ingestión oral, penetran la BHE y responden a

cambios de glucosa o citoquinas en suero, que homeostáticamente controlan el eje HPA y regulan la disponibilidad de cortisol (Whitehouse, 2011). La distinción entre estos GC sintéticos y los fisiológicos es clave, ya que tienen diferentes afinidades por sus receptores de unión. En concreto, en el SNC existe un escenario complejo, ya que se ha descrito que para los GC sintéticos es difícil cruzar la BHE, debido a la presencia de transportadores de resistencia a multifármacos (De Kloet et al., 1998), ya que estos esteroides constituyen un grupo heterogéneo de componentes. Es por ello por lo que se ha sugerido que los estudios *in vivo* que utilizan GC sintéticos deberían utilizar dosis suficientemente altas para saturar estos transportadores si se desean efectos en el SNC. La Tabla 3 representa los principales efectos terapéuticos que han sido demostrados en la clínica para los GC.

**Tabla 3.** Efectos terapéuticos de los GC sintéticos (adaptada de Vandewalle et al., 2018 y Timmermans et al., 2019).

| | |
|---|---|
| **Inflamatorios** | Inhibición NF-KB<br>Supresión de ciclooxigenasa<br>Antiinflamatorios / Proinflamatorios (controversia) |
| **Inmunológicos** | Inmunosupresión |
| **Endocrino-metabólicos** | Gluconeogénesis<br>Degradación proteica<br>Redistribución de la grasa |
| **Neurológicos** | Alteración síntesis mediadores<br>Inhibición neuroinflamación / Neurotóxicos (controversia) |
| **Psicológicos** | Regulación del humor |
| **Musculo-esqueléticos** | Menor actividad fibroblástica<br>Inhibición de condrocitos<br>Inhibición de osteoblastos |
| **Cardiovasculares** | Disminución de la permeabilidad capilar |

Así, a día de hoy, se conoce que la exposición crónica a GC inhibe tanto el sistema inmune innato como el adaptativo, reduciendo el nivel de leucocitos circulantes en sangre y la producción de una gran variedad de citoquinas proinflamatorias, incluyendo IL-1β y TNF-α (De Bosscher et al., 2003). En el caso de IL-1, IL-6 e IFN-γ, los GC suprimen no sólo la producción del mediador (Munck et al., 1984; Guerne et al., 1990), sino que a la vez inducen sus receptores

(Snyers et al., 1990). Tales efectos opuestos en el mismo mediador podrían parecer una anulación de uno a otro. Este no es el caso, sin embargo. Las acciones permisivas y supresoras pueden conllevar una unión para regular los mecanismos de defensa en un gran rango de niveles fisiológicos de GC.

En la mayoría de casos de exposición crónica disminuye la respuesta inmune celular, reafirmando así las propiedades inmunosupresoras descritas; no obstante, en ocasiones puede ocurrir lo contrario y aumentar dicha inmunidad (Bowers et al., 2008). Por ello resulta importante la duración de la exposición al GC para obtener una u otra respuesta del sistema inmune. En realidad, desde hace tiempo, los estudios en animales indicaban que los efectos de los GC son complejos y que solamente tienen efecto protector bajo algunas circunstancias (McEwen et al., 1992), habiéndose sugerido que podían aumentar aspectos de la inflamación en el SNC (Sorrells et al., 2009), lo cual puede exacerbar la muerte neuronal. El efecto será antiinflamatorio o proinflamatorio dependiendo de factores como dosis, tiempo, duración de la exposición y tipo de GC (Sorrells et al., 2009).

En definitiva, existen pocas clases de fármacos que presenten más paradojas que el cortisol y los GC, antiinflamatorios: tan efectivos para suprimir la inflamación, pero con un potencial intrínseco tan dañino; tan esenciales para preservar la vida, pero capaces de dañar a muchos de los órganos corporales; tan centrales para la práctica clínica durante los 60 últimos años como fármacos de elección, pero actualmente sólo utilizados cuando otros antiinflamatorios han fallado.

#### i. Efectos en el SNC

Los GC ejercen influencia en la actividad neuronal alterando la síntesis, metabolismo y nivel de receptores de neurotransmisores y neuromoduladores, que incluyen la biosíntesis de 5-HT y noradrenalina en determinadas regiones encefálicas, especialmente en el hipocampo y el hipotálamo (McEwen et al., 1986).

La DEX y la prednisona son los fármacos más empleados en clínica para el control de la neuroinflamación aguda (Whitehouse, 2011). Se han utilizado altas dosis de ambos esteroides para alcanzar la concentración que se requiere en el SNC (Marchi et al., 2011). La acción farmacológica de la DEX incluye la reducción de citoquinas y el incremento de expresión de proteínas de unión *tight* en membranas de células endoteliales basolaterales, haciéndolas menos permeables (Forster et al., 2006). La DEX penetra en el encéfalo principalmente a través de difusión ventricular. Evidencia de esto es que tras su administración, se une a células gliales

que rodean los ventrículos (Nestler et al., 1981). En este sentido, es probable que la DEX prevenga el paso de patógenos desde la sangre al parénquima encefálico al mismo tiempo que hace que disminuya la expresión de citoquinas como TNF-α e IL-1β, contribuyendo así a la reducción del daño neuronal.

Por un lado, se han atribuido un papel neuroprotector a estos fármacos (sobre todo a la DEX) en distintos modelos de estudio. Así, se ha demostrado que la DEX reduce la neurotoxicidad en cultivos mixtos de neuronas y astrocitos promoviendo la neuroprotección a través de la estimulación de la glutamina sintetasa astrocítica (Debroas et al., 2015). También Ramesh et al., 2017 observaron un efecto neuroprotector de la DEX en la neuroinflamación producida en la borreliosis, sugiriendo que podría servir como diana terapéutica para limitar la neuroinflamación. En otro estudio, la DEX previno los signos clínicos y neurológicos (déficits motores y daño neurovascular) producidos por una toxina de *E. coli* y lipopolisacárido (LPS) en ratones, demostrando ser un fármaco efectivo (Pinto et al., 2017). También Fischer al., 2019 han atribuido un impacto positivo en la neuroinflamación y un efecto terapéutico de los GC en esclerosis múltiple, ya que consiguieron observar una polarización M2 de la microglía (fenotipo antiinflamatorio). Muy recientemente, Nerius et al., 2020 han demostrado que la terapia con GC se asocia con un menor riesgo de demencia, sobre todo administrados vía tópica o inhalatoria.

Sin embargo, por otro lado, se ha mostrado que los GC también pueden ejercer efectos neurotóxicos, como en EA, en la que se ha observado inducción de neurodegeneración y efectos proinflamatorios en corteza frontal e hipocampo de ratones tras la exposición crónica a GC (Hu et al., 2016). También Zhang et al., 2017 observaron que esta exposición crónica estaba implicada en el daño neuronal del hipocampo. Además, se conoce que los GC pueden acelerar algunos aspectos del envejecimiento del encéfalo (Landfield et al., 1978) y que puede contribuir a la pérdida neuronal senescente (Reagan and McEwen, 1997). Otros investigadores han examinado los efectos del tratamiento con GC a largo plazo en primates (Sapolsky et al., 1990), observando que, tras un año de tratamiento, el daño neuronal se producía principalmente en las células piramidales CA3 del hipocampo. Precisamente en el hipocampo es donde más estudios se han realizado, llegándose a observar degeneraciones en la morfología astrocítica en ratas adultas cuyas madres habían sido tratadas con DEX durante la gestación. Por lo tanto, ésta tiene un efecto en la remodelación y plasticidad astrocítica en el hipocampo (Shende et al., 2015).

En resumen, los efectos en el SNC de los GC siguen siendo actualmente una cuestión controvertida. El tiempo de exposición a los GC parece ser clave en las acciones opuestas de dichas hormonas esteroideas (Bellavance and Rivest, 2014). Deberían tenerse en cuenta factores relevantes como la duración del tratamiento, dosis, ruta de administración y tipo de GC (Sorrells et al., 2009). Otro factor relevante a considerar cuando se utilizan estos fármacos es la resistencia adquirida, que se manifiesta generalmente por la ausencia de respuesta al tratamiento, bien descrita en artritis reumatoide (Kirwan, 2007).

**ii. Efectos adversos**

Ya desde la década de los 50, se observó que con grandes dosis y usado a largo plazo, los efectos terapéuticos de la cortisona tenían efectos secundarios indeseables, como la excesiva retención de agua y sal, acidez gástrica incrementada y psicosis. Sin embargo, hay pocas revisiones dedicadas a los mecanismos que subyacen a los efectos tóxicos adversos que estos fármacos hormonales pueden provocar. Efectivamente, tras un tratamiento con esteroides a largo plazo y a grandes dosis, se han descrito efectos secundarios graves, que han sido confirmados en repetidas ocasiones con la experiencia clínica (Hoes et al., 2009). Entre éstos se incluyen: exacerbación o aparición de diabetes *mellitus*, reflujo gastroesofágico, osteoporosis, osteonecrosis no traumática, síndrome de Cushing, hipertensión, cambios de comportamiento y neurodegeneración (Howell and Muglia, 2006). Es por ello por lo que se ha limitado su uso en el tratamiento de neuropatologías crónicas (Whitehouse, 2011; Danilczuk et al., 2001). Dichos efectos secundarios son difíciles de evitar, ya que la mayoría de esteroides antiinflamatorios e inmunosupresores se unen a receptores de esteroides que están expresados ubicuamente, con lo cual se afecta la funcionalidad de muchos tipos celulares diferentes, implicados en procesos biológicos importantes y no sólo en la inmunidad innata y adaptativa (Tischner and Reichardt, 2007). Por lo tanto, actualmente existe la necesidad urgente de mejorar esta terapia.

Algunas de estas reacciones adversas son controvertidas, como la teratogenicidad o la destrucción del tejido linfoide, que tiene como consecuencia grave el incremento de la susceptibilidad a infecciones secundarias (Whitehouse, 2011). El hecho de que se potencie el catabolismo de macromoléculas daña los mecanismos de reparación dérmica, afectando a la renovación de glicosaminoglicanos y ácido hialurónico esenciales para las funciones normales de la piel, defensas inmunológicas y reparación de heridas (Weindl et al., 2004; Sorrells and Sapolsky, 2007). En la Tabla 4 se resumen los principales efectos adversos provocados por los GC.

**Tabla 4.** Efectos adversos de los glucocorticoides usados de forma prolongada y a altas dosis (Adaptada de Hoes et al., 2009).

| | |
|---|---|
| **Músculo - esqueléticos** | Osteoporosis,<br>Osteonecrosis,<br>Miopatía |
| **Endocrinos/metabólicos** | Intolerancia a la glucosa (diabetes)<br>Redistribución de la grasa y peso corporal |
| **Cardiovasculares** | Dislipidemia<br>Aterosclerosis<br>Edema<br>Hipertensión |
| **Dermatológicos** | Atrofia cutánea<br>Hirsutismo<br>Alopecias |
| **Oftalmológicos** | Cataratas<br>Glaucoma |
| **Gastrointestinales** | Úlceras gástricas<br>Pancreatitis |
| **Infecciosos** | Infecciones bacterianas de la piel |
| **Psicológicos / Cambios de comportamiento** | Cambios de humor y comportamiento |
| **Neurológicos** | Dolor de cabeza<br>Mareos |

# IV. OBJETIVOS

# IV.OBJETIVOS

El objetivo general de esta tesis doctoral es contribuir al conocimiento del papel que desempeña el sistema inmunológico del hospedador en el progreso neurodegenerativo de las enfermedades priónicas mediante el estudio de las principales poblaciones celulares implicadas en la defensa del sistema nervioso central, astrocitos y microglía, así como de diferentes marcadores de reacción inflamatoria liberados por ellas.

En base a estudios epidemiológicos previos sobre el efecto protector del uso de antiinflamatorios, se pretende evaluar el impacto real de dichos fármacos en el progreso natural de la neurodegeneración. Para ello se plantea valorar, tras un tratamiento con glucocorticoide, la reacción inmunológica a nivel encefálico a través de diversos marcadores inflamatorios en animales afectados de forma natural de scrapie (prototipo de las enfermedades priónicas) con el objetivo de poder extrapolar resultados a otras enfermedades priónicas y prion-*like*. El objetivo final es determinar si la modulación de la respuesta neuroinflamatoria a través de las células gliales mediante fármacos antiinflamatorios podría llegar a convertirse en una estrategia terapéutica de investigación frente al proceso neurodegenerativo.

Los objetivos específicos que se plantean son los siguientes:

1. Profundizar en los factores celulares y moleculares relacionados con el sistema de neuroprotección en relación con el proceso neurodegenerativo evaluando la expresión local de varias citoquinas y/o sus receptores en el progreso del scrapie natural ovino mediante el estudio de animales en diferentes estadios de la enfermedad: preclínico, clínico y terminal.

2. Desarrollar un modelo de tratamiento crónico con un glucocorticoide sintético (dexametasona) en el modelo ovino para poder evaluar posteriormente el efecto del fármaco sobre parámetros clínicos y neuropatológicos de ovejas que están afectadas de scrapie.

3. Analizar la influencia del tratamiento con dexametasona sobre la respuesta inmunológica del individuo a nivel encefálico en el desarrollo y/o propagación de la neurodegeneración usando el modelo natural de enfermedad priónica, describiendo específicamente las variaciones en la intensidad y distribución, así como la morfología

de las células astrogliales y microgliales en los animales afectados de la enfermedad que han sido tratados.

4. Describir los posibles cambios en la relación de interacción existente entre las dos poblaciones gliales estudiadas mediante la liberación de citoquinas, con especial énfasis en aquellas citoquinas que estén directamente implicadas en dicha comunicación bidireccional. Para ello, se valorará el efecto de la terapia antiinflamatoria e inmunosupresora con dexametasona en la expresión de diferentes citoquinas neuroinflamatorias y/o sus receptores en distintas áreas del sistema nervioso central de animales con sintomatología clínica característica de la enfermedad.

5. Determinar si el efecto del tratamiento antiinflamatorio valorado en los objetivos 3 y 4 difiere cuando se inicia en fases más tempranas de la enfermedad (estadio preclínico) y por lo tanto, del proceso de neurodegeneración.

# V. RESULTADOS Y DISCUSIÓN

# ESTUDIO Nº 1:

## IMMUNOHISTOCHEMICAL STUDY OF NEUROINFLAMMATORY CYTOKINES IN THE PROGRESS OF PRION DISEASES

# V. RESULTADOS Y DISCUSIÓN

## ESTUDIO Nº 1: IMMUNOHISTOCHEMICAL STUDY OF NEUROINFLAMMATORY CYTOKINES IN SCRAPIE AFFECTED SHEEP AT DIFFERENT CLINICAL STAGES


**ABSTRACT**

Nowadays, central nervous system (CNS) immunology is acquiring a crucial role in prion diseases and other neurodegenerative disorders since neuroinflammatory mechanisms are known to have a role in the neurodegenerative process associated with all these diseases. Consequently, a host innate immune response mediated by cytokines is probably involved in mechanisms contributing to neurodegeneration. The aim of this study was to assess the staining intensity of IL-1, IL-1R, IL-2R, IL-6, IL-10R, TNFαR and IFNγR by immunohistochemistry (IHC) in Purkinje cells of cerebellum of sheep affected by natural scrapie at different clinical stages (preclinical, clinical and terminal) in order to evaluate whether the intensity of their immunostaining vary throughout disease progress.

IHC results showed evident changes in the expression of most of the assessed markers in scrapie affected sheep compared to healthy controls. In particular, IL-1 and IL-6 intensity increased in a significant way in clinical and terminal sheep, while it was the contrary for IL-1R and IL-2R, being these markers diminished in those two stages. Moreover, IL-10R and TNFαR intensity substantially increased from preclinical stage onwards, being the change in TNFαR in all stages of scrapie highly significant compared to healthy sheep. Finally, no evident changes were appreciated for IFNγR over the course of scrapie, only a subtle decrease in intensity in both clinical and terminal stage.

In summary, this preliminary study confirms that a complex network of cytokines is involved in the pathogenesis of natural scrapie, thus suggesting a relevant immunological component in prion diseases. In the future, this study is planned to be extended to both other inflammatory mediators and brain regions, paying particular attention to their release by astroglial and microglial populations. Understanding cytokine action in scrapie and other prion and *prion-like* diseases could lead to novel therapeutic strategies.



**Key words:** cytokines, neuroinflammation, progress, prion diseases, scrapie.

**INTRODUCTION**

Transmissible Spongiform Encephalopathies (TSE) or Prion diseases are a group of neurodegenerative diseases associated with misfolded protein deposit affecting both animals and humans. Scrapie is an endemic TSE in many countries worldwide naturally affecting sheep, goats and mouflons (Jeffrey and González, 2007). As this prion disease has been widely studied, it is the natural model used as a prototype of this group of diseases. However, not many studies have been focused on the naturally occurring disease in sheep. Thus, there is a renewed interest in studying the natural model in order to provide reliable conclusions.

Nowadays, central nervous system (CNS) immunology is acquiring an important role in these and other neurodegenerative disorders (Aguzzi et al., 2013; Kang et al., 2016; Richards et al., 2016; Mabbott, 2017). In contrast to the theory about brain is an 'immune privileged place (Barker and Billingham, 1977), neuroinflammation hypothesis postulates that neuroinflammatory mechanisms have a role in the neurodegenerative process associated with prion diseases (Burwinkel et al., 2004; Pasquali et al., 2006; Marcos-Carcavilla et al., 2007; Servida et al., 2007).Thus, an innate immune response carried out by dendritic cells, macrophages and microglia, has been described during the course of scrapie infection (Beringue et al., 2000; Marella and Chabry, 2004).

A growing body of evidence indicates that prion protein deposit in a determined anatomical area may act as stimulus leading to glial activation with the consequent *in vivo* production of cytokines (Williams et al., 1994; Cunningham et al., 2005).These signalling proteins can act centrally to activate neural aspects of the immune response such as the hypothalamic-pituitary axis, thus serving as a regulatory signal between the immune system and the CNS (Benveniste, 1992). Thus, their involvement in CNS pathology is a growing area of clinical research (Richards et al., 2016; Efremova et al., 2017). Consequently, the host immune response mediated by cytokines is probably involved in neuroinflammatory mechanisms contributing to neurodegeneration (DiSabato et al., 2016; Ransohoff, 2016; Kempuraj et al., 2016). In particular, the actual role of prion protein - induced glia activation and subsequent cytokine secretion during the infection is still incompletely understood.

Overall, the presence of cytokines has been assessed in several human neurodegenerative diseases demonstrating that they are produced by different cellular types, glial cells among them (Dickson et al., 1991; Dickson et al., 1993; Liberski et al., 1995; Veerhuis et al., 2002). Some previous studies have proposed that cytokines and other acute phase proteins may contribute to the development of chronic neuropathologies such as Alzheimer disease (AD)

(Berkenbosch et al., 1992; Akiyama et al., 2000; Abbas et al., 2002; Janelsins et al., 2005; Sastre et al., 2006; McGeer et al., 2016). Specifically regarding prion diseases, an altered profile of inflammatory intermediaries has been evidenced in some experimental murine models (Liberski et al., 1990; Williams et al., 1994; Campbell et al., 1994; Liberski et al., 1995; Kordek et al., 1996; Cunningham et al., 2002; Brown et al., 2003; Schultz et al., 2004; Thackray et al., 2004; Tribouillard-Tanvier et al., 2009).However, the local inflammatory response has been scarcely dealt (Williams et al., 1994; Cunningham et al., 2005), even no treated in tissues from natural model individuals along the progress of neurodegeneration.

Some previous studies about scrapie have been focused on the cerebellum based on the fact that it is a particularly vulnerable brain region to insults and abnormalities in the cerebellum are usually easy to recognize (Sarna et al., 2003). In addition, it is an encephalic area which has been proposed as a pseudo reference region to detect neuroinflammation (Lyoo et al., 2015). Specifically, Purkinje cells have been concluded from our previous studies (Hernández et al., 2014; Sarasa et al., 2015) to be the most damaged type of neurons as well as to play a relevant role in the neurodegenerative progress.

Thus, the aim of this study is to assess the local expression of several both cytokines and cytokine receptors in this specific affected encephalic area (cerebellum) by using natural scrapie animals at different clinical stages (preclinical, clinical and terminal). To evaluate whether the levels of their expression at brain level vary throughout disease progress is intended here.

## MATERIAL AND METHODS

This study was approved by the Ethical Committee for Animal Welfare from the University of Zaragoza (Reference number: PI 41/16).

*SAMPLES*

Samples corresponded to 31 sheep. On one hand, 23 from naturally acquired scrapie sheep at different clinical stages: 7 preclinical (when animal did not present clinical signs but provided positive results for PrPsc detection by lymph reticular biopsy), 8 clinical and 8 terminal (when the animal was exhaustively debilitated and prostrated). On the other hand, 8 healthy animals were used as negative controls. Different PrP genotypes (ARQ/ARQ, ARQ/VRQ and VRQ/VRQ) were included in the study.

After euthanasia, brains were fixed by immersion in 10 % formaldehyde and paraffin-embedded.

Fixed sections corresponding to cerebellar sagital samples included the granular, Purkinje and molecular layers and white matter for all samples. Cerebellar slices (4 µm) were incubated at 56 °C overnight before subsequent treatment.

*IMMUNOHISTOCHEMISTRY*

Immunohistochemical (IHC) protocols using specific monoclonal antibodies were performed to detect some neuroinflammatory cytokines (IL-1, IL-1R, IL-2R, IL-6, IL-10R, TNF-αR and IFN-γR) in cerebella from the 31 sheep mentioned above.

After heat treatment (96ºC for 10–20 min, depending on the primary antibody), endogenous peroxidase blocking (10 min) was applied on all samples. Then, slices were incubated with the respective primary antibody: Anti-IL1RN (Sigma, Sweden); Monoclonal mouse Anti-Human CD30 (DAKO, Denmark); CD25 Monoclonal Antibody, IL-10RA Monoclonal Antibody, IFNGR1 Polyclonal Antibody, IL-1 alpha Polyclonal Antibody, IL-6 Monoclonal Antibody (ThermoFisher, Denmark) following Manufacturer's instructions (overnight at 4ºC; see Table 1 for further information). Secondary antibody was added depending on the primary antibody used: Envision Anti-Mouse or Envision Anti-Rabbit (DAKO, Denmark) for 30 min and DAB visualisation system was performed (10 min). Slides were counterstained with haematoxylin, dehydrated and mounted with DPX mounting media (DAKO, Denmark).

**Table 1.** Details about the primary antibodies used for each marker assessed including specific pre-treatment for their detection by immunohistochemistry in paraffin embedded samples.

| Antibody | Antigen | Type | Dilution | Specific pretreatment | Source |
|---|---|---|---|---|---|
| IL-1 alpha | IL-1 | Polyclonal | 1:100 | Formic acid 15 min 96 ºC 20 min | ThermoFisher |
| Anti-IL-1RN | IL-1R | Polyclonal | 1:100 | Formic acid 15 min 96 ºC 20 min | Sigma |
| IL-2R.1 | IL-2R | Monoclonal | 1:1.000 | 96 ºC 10 min | ThermoFisher |
| 8H12 | IL-6 | Monoclonal | 1:20 | Formic acid 15 min 96 ºC 20 min | ThermoFisher |
| OTI1D10 | IL-10R | Monoclonal | 1:250 | Formic acid 15 min 96 ºC 20 min | ThermoFisher |
| Ber-H2 | TNFαR | Monoclonal | Ready to use | 96 ºC 15 min | Dako |
| IFNGR1 | IFNYR | Polyclonal | 1:200 | 96 ºC 20 min | ThermoFisher |

Immunolabelling morphology was specifically examined focusing on Purkinje cell layer and subjectively scored using light microscopy according to the intensity and the area over which the labelling extended to; i.e.: 0 = absence of labelling; 4 = intense brown labelling over most areas.

*STATISTICAL ANALYSIS*

The normal distribution of values was analyzed with the Kolmogorov–Smirnov test. Statistical analysis of the intensity data between groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test using the SPSS software (SPSS Statistics for Windows, Version 17.0).

Graphics were performed using the GraphPad software (GraphPad Prism, Version 6.01). All data were expressed as mean values ± SEM. Differences between groups were considered statistically significant at * $p < 0.05$ and ** $p < 0.01$.

**RESULTS**

*IMMUNOHISTOCHEMISTRY*

The IHC results showed evident changes in the expression of most of the assessed markers in crapie affected sheep compared to healthy controls. Purkinje neurons were often stained regardless the cytokine or respective receptor assessed. In general, these cells usually exhibited immunostaining more frequently in scrapie affected sheep (in all stages) than in control sheep. Our immunohistochemical results revealed that Purkinje cells highly express different neuroinflammatory cytokines (Figure 1).

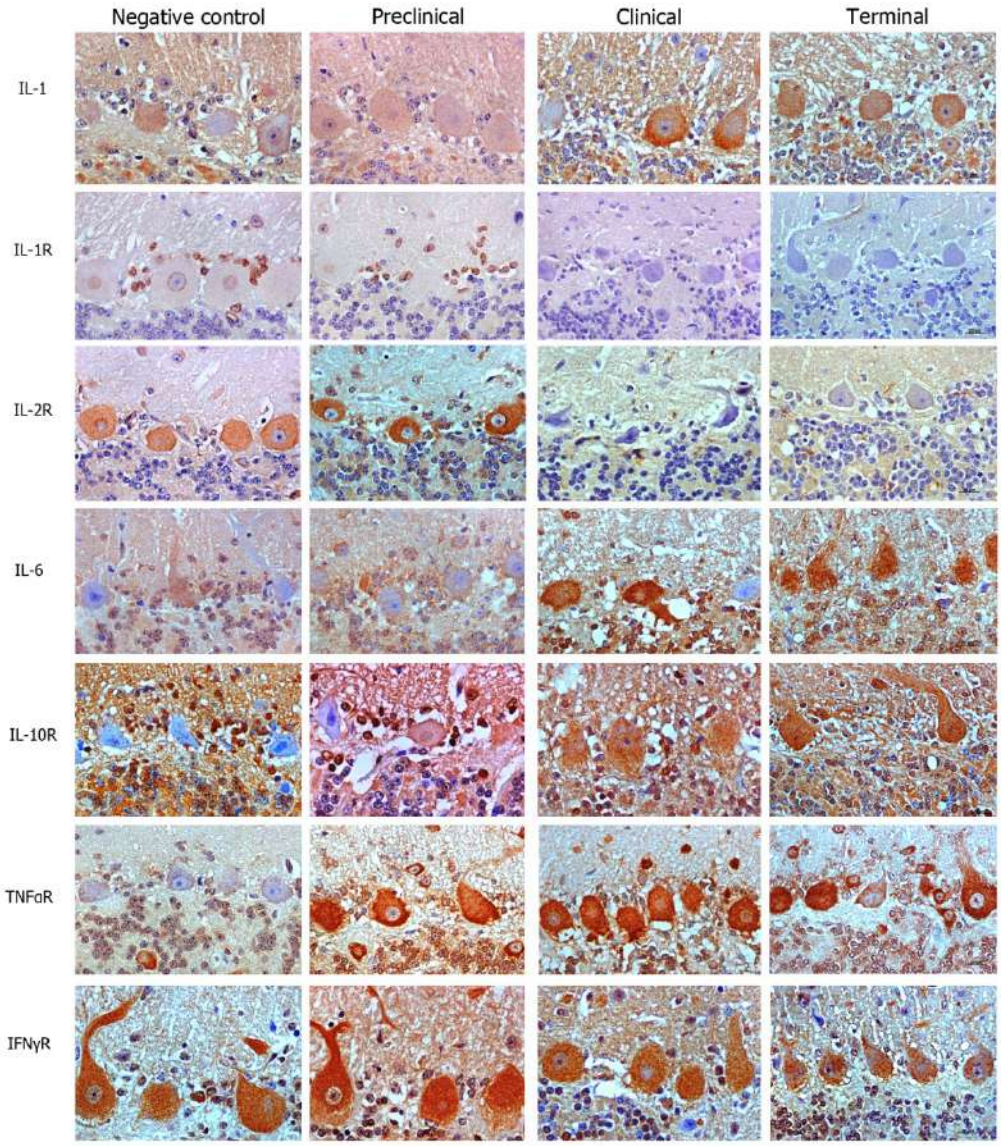


**Figure 1.** Images corresponding to Purkinje cell layer from cerebella corresponding to healthy, preclinical, clinical, and terminally scrapie affected sheep immunostained with antibodies against different neuroinflammatory cytokines. Note the spongiform morphology characteristic of scrapie in the cerebellum of clinical and terminal sheep. Scale bars = 20 µm.

A basal expression level of IL-1 and IL-6 was observed in Purkinje cells of negative controls, but these pro-inflammatory cytokines seemed to significantly increase their expression as scrapie evolution progressed, reaching the maximum intensity in Purkinje cells from animals at terminal stage of the disease (Figure 1). In fact, statistically significant differences were found between negative controls and terminal stage of scrapie for IL-1 (** $p < 0.01$) and IL-6 (* $p < 0.05$) (Figure 2).

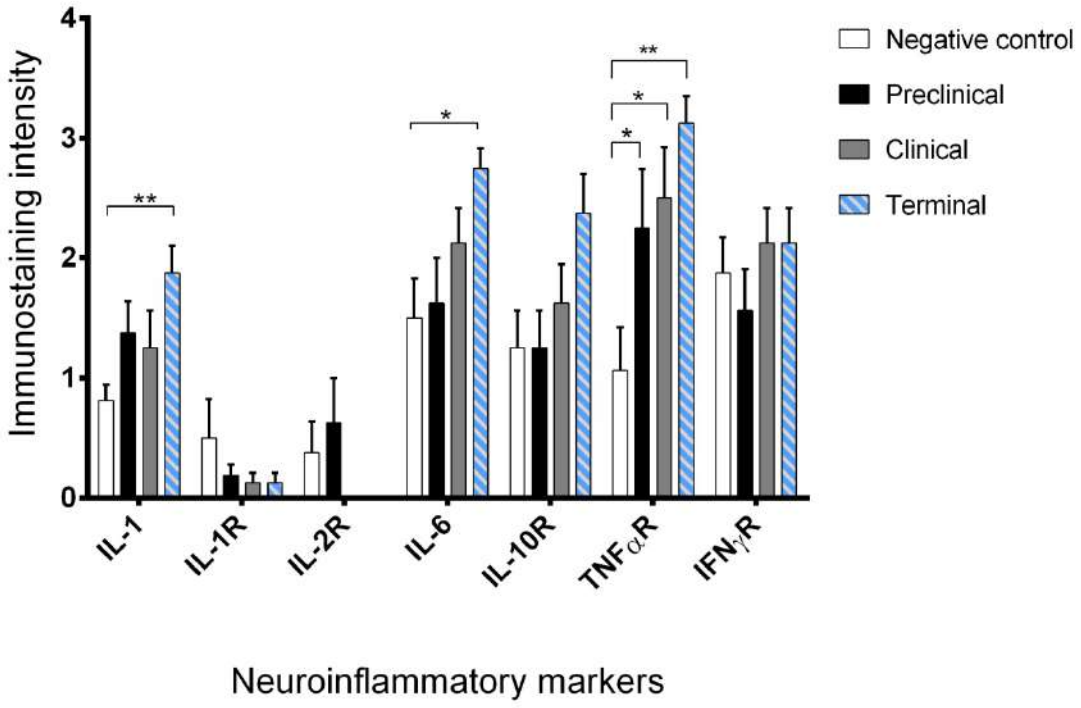


**Figure 2.** Intensity values for each neuroinflammatory marker subjectively scored by immunohistochemistry. Data are expressed as mean ± SEM. Differences between groups are considered statistically significant at *$p$ < 0.05 and **$p$ <0.01. Statistically significant differences were found between immunostaining intensity at terminal stage of scrapie and negative controls for IL-1 (**$p$ < 0.01), IL-6 (*$p$ < 0.05) and TNF-αR (**$p$ < 0.01) by Bonferroni *post-hoc*. In addition, significant differences were also found for TNF-αR between control and the clinical and terminal stages of the disease (*$p$ < 0.05).

A basal level of cytokine receptors was evidenced in negative control sheep. Levels of both IL-1R and IL-2R were similar each other and IL-10R and TNFαR as well. IFNγR expression level was higher than the other receptors in such negative controls (Figure 1).

A noticeable and statistically significative increase of TNFαR in preclinical stage of scrapie was observed compared to healthy sheep (* $p$ < 0.05) (Figures 1 and 2). In the same line, IL-2R and IL-10R raised its expression slightly in that stage, not being so evident. However, IL-1R behaved different to the rest; this receptor diminished its expression over the disease course, reaching a minimum intensity in terminal stage of scrapie (Figure 1).

The clinical phase of the disease was characterized by an increase in the expression of TNFαR, IL-10R, IFNγR and a substancial decrease of IL-1R and IL-2R compared to preclinical sheep. Moreover, comparing clinical and healthy status, there was significant differences for TNFαR (* $p$ < 0.05) (Figure 2).

Sheep with scrapie in terminal stage with respect to clinical sheep expressed with a much higher intensity TNFαR (** $p < 0.01$) (Figure 2) and IL-10R in Purkinje cells of cerebellum, and maintained the intensity of expression of IFNγR, IL-1R and IL-2R. In terms of intensity, most clinical and terminal sheep showed a widespread pattern for IL-6, IL-10R, TNFαR and IFNγR (Figure 1).

Comparing the cell morphology among the neuroinflammatory markers assessed here, the cytokine profiles were slightly different. In general, for all markers, immunoreactive cells showed no morphological changes throughout the course of scrapie (Figure 3). On one hand, both IL-1 and IL-6 staining in clinical sheep followed an invariably pattern, consistent in Purkinje cell intracitoplasmatic labelling, although it did not appear so evident in their non-infected counterparts. Several immunopositive cells for IL-1R in negative and preclinical stage exhibited a glial staining likely corresponding to astrocytes (Figure 3A). On the other hand, IL-10R was expressed as little intracitoplasmatic spots in Purkinje cells of negative control sheep (Figure 3B). In addition, IL-2R staining during clinical and terminal stage was granular and expressed by pleomorphic cells that might be easily identified with some specific antibodies, mainly located in white matter; this marker also was present on some blood vessels with thick walls but not in Purkinje cells in those stages (Figures 3C and 3D). TNFαR showed more defined cell morphology, being expressed in not only Purkinje cells but in other cell types in granular and molecular layer in all stages (Figure 3E). For IFNγR, the immunostaining pattern appeared more uniform in Purkinje cell dendritic spines, mainly in negative controls and preclinical stage (Figure 3F).

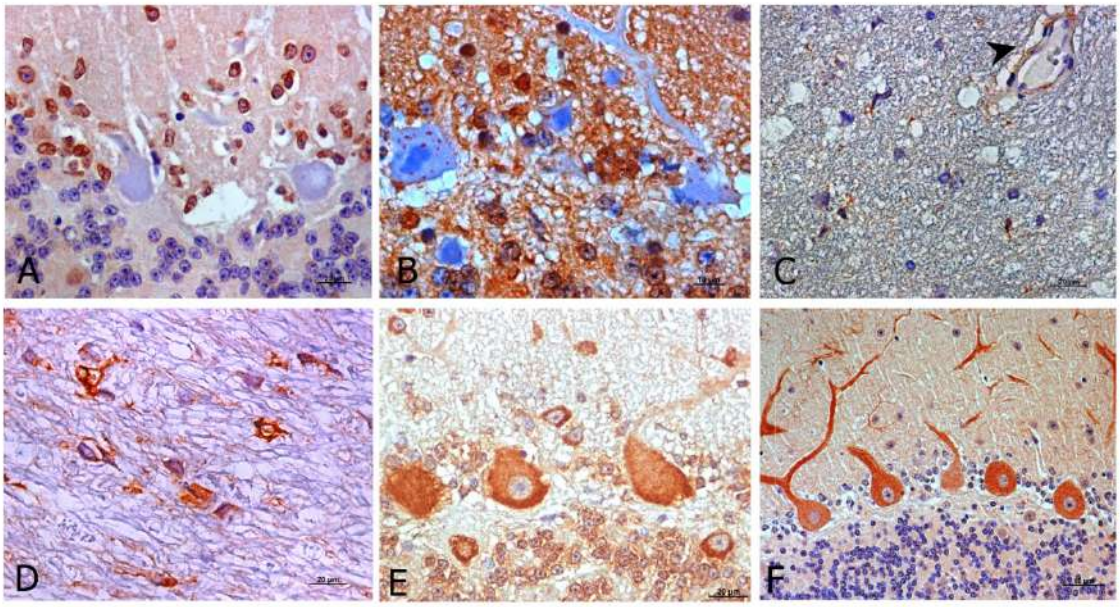


**Figure 3.** Outstanding morphological findings provided by light microscopic examination after IHC for detection of neuroinflammatory cytokines (brown; hematoxylin counterstaining, blue). **(A)** Several immunopositive cells for IL-1R at preclinical stage exhibited staining likely corresponding to astrocytes **(B)** IL-10R was expressed as intracitoplasmatic spots in Purkinje cells from negative control sheep. **(C and D)** In addition, IL-2R immunostaining during clinical (C) and terminal stages (D) was frequently expressed by pleomorphic cells as well and it was also present on some blood vessels with thick walls (C, arrow). **(E)** TNFαR was expressed not only in Purkinje cells but also in other cell types in granular and molecular layer in all stages (E, preclinical). **(F)** For IFNγR, the immunostaining pattern appeared more uniform in Purkinje cell dendritic spines, mostly in negative controls as well as preclinical stage of scrapie (F, preclinical). Scale bars: 10 µm in (B); 20 µm in (A, C, D, E); 50 µm in (F).

In summary, it could be said that Purkinje cells in cerebellum express cytokine receptors with higher or lower intensity in a different way, being TNFαR and IL-10R which raise in a substancial way with the progress of the disease, while, on the contrary, IL-1R keeps diminishing its expression in such cells over the scrapie course in naturally infected sheep. For IL-2R, there was little variation regarding the distribution pattern among all the positive sheep and between them and healthy controls. Most of the differences for this receptor consisted of higher labelling intensity of Purkinje cells in cerebellum of preclinical sheep compared to negative controls. Finally, regarding IFNγR, no evident changes were appreciated, only a subtle increase in expression in both clinical and terminal stage of scrapie compared to negative controls (Figure 1).

**DISCUSSION**

This study reports possible markers related to inflammation using scrapie as natural model of prion disease, with the aim to investigate the role of the host immune system in the disease progress. Not many studies have focused on the host immune response in prion diseases, since it has always been accepted that these diseases do not display the typical immune response observed in other infectious diseases (Aguzzi, 2003).

In this study, we used immunohistochemistry to determine the intensity level and distribution of IL-1, IL-1R, IL-2R, IL-6, IL-10R, TNFαR and IFNγR in the cerebellum of naturally scrapie infected sheep through the progress of neurodegeneration (at preclinical, clinical and terminal stage of the disease). Among all the cytokines and its receptors, we decided to focus on these markers because much data have been collected and all the authors agree that those cytokines could be significant factors determining the pathogenesis of neurodegeneration in scrapie (Burwinkel et al., 2004; Pasquali et al., 2006; Marcos-Carcavilla et al., 2007; Servida et al., 2007).With this study we pretend to ascertain whether these cytokines can act directly on Purkinje neurons.

There are several studies in prion diseases in which the expression of proinflammatory cytokines have been described (Campbell et al., 1994; Williams et al., 1994; Kordek et al., 1996; Williams et al., 1997), often simultaneously with the onset of clinical stage. However, our profile of cytokines is atypical compared to the results obtained in those studies, since we have already detected a raise in some cytokines even at preclinical stage of scrapie. The differences might be due to the fact that none of these previous studies have focused on the progress of neurodegeneration, and even less by using a natural model of prion disease.

Our immunohistochemical study showed that Purkinje cells express high levels of IL-10R, TNFαR and IFNγR at clinical and terminal stages of scrapie, indicating that their substrates, IL-10, TNFα and IFNγ, could act directly on Purkinje neurons to alter their physiological properties.

For TNFαR antibody, a staining pattern was inconsistently observed at preclinical stage of disease, demonstrating that cytokine immunoreactivity was present before the clinical signs started, which is in agreement with the report by Williams et al., 1997. However, from preclinical stage onwards, a significant increase in immunostained Purkinje cells was detected, lasting until the terminal stage of the disease. Provided that TNFα has been reported to have

either neuroprotective or neurotoxic roles (Scherbel et al., 1999; Mabbott et al., 2000), the significance of this is unclear yet.

Cunningham et al., 2005 demonstrated an exacerbation of IL-1 in a mouse model of prion disease. Likewise, we have shown that this cytokine is highly expressed in ovine scrapie. Actually, it has been described the presence of an impaired response of cytokines in mice orally infected with scrapie, imitating the natural route of entry (Romero-Trevejo et al., 2010). In the same line, IL-1 gene expression has been related to susceptibility to scrapie in sheep, showing differences in the expression of this cytokine in cerebellum, but not in spleen (Marcos-Carcavilla et al., 2007). Similar to human studies in AD (Griffin et al., 1995; Akiyama et al., 2000; Griffin and Mrak, 2002; Sastre et al., 2006), we have observed that IL-1 is overexpressed in preclinical stage of ovine scrapie compared to negative controls. Likewise, the early overexpression of IL-1 has been suggested as a candidate to contribute to the development of scrapie (Brown et al., 2003).

In our study in sheep, several IL-1R immunopositive cells had morphology consistent with that of astrocytes, in agreement with reports of IL-1 immunostaining in murine models (Williams et al., 1994; Kordek et al., 1996; Williams et al., 1997; Brown et al., 2003).

To date, there are studies indicating that IL-6 can have both neurotrophic and neurotoxic effects on CNS neurons (Allan and Rothwell, 2001).In addition, IL-6 has been reported to exert trophic effects on glial cells, particularly oligodendrocytes (Barres et al., 1993).In our case, we have found that this cytokine increased significantly its expression in Purkinje cells, reaching the maximum intensity in terminal stage of scrapie. This is in agreement with previous reports, in which overexpression of IL-6 itself was demonstrated to produce neurologic disease in mouse, activating astrocytes and microglia (Campbell et al., 1993).Also, IL-6 was shown to activate astrocytes *in vitro* (Hafiz and Brown, 2000). Actually, Gabay, 2006 proposed blocking of IL-6 as a possible treatment against chronic inflammatory diseases.

One striking finding was that IL-1R, IL-2R, and IFNγR were not as so increased in scrapie. In fact, in our study both IL-1R and IL-2R were decreased in clinical animals compared to controls. From preclinical stage onwards, there was a progressive decrease in the expresssion of IL-2R by immunostained Purkinje cells. No expression of this receptor was observed in Purkinje cells in clinical and terminal stages of ovine scrapie. This raises the possibility that IL-2 may well play a role in scrapie, in contrast to what other authors postulated (Brown et al., 2003).Taken together, these data mean that a role for IL-2 in TSE neuropathology cannot be ruled out.

It has been described that there is an accelerated prion disease in absence of IL-10, suggesting that the anti-inflammatory cytokine IL-10 is neuroprotective (Thackray et al., 2004). In comparison to that experiment, we have observed that its receptor, IL-10R, is expressed in a higher intensity over scrapie progress. This discrepancy in results may be due to the lack of uniformity between studies.

IFNγR expression showed only subtle changes between negative and scrapie affected sheep, not being so evident. On the basis of those results, we suggest that this receptor do not play a significant role in ovine scrapie. Nonetheless, it has been described that prolonged expression of its substrate, IFNγ, in CNS can lead to neuronal and glial cell damage (Corbin et al., 1996).

In spite of there are previous reports which did not detect an association between the upregulation of any proinflammatory cytokines and the course of the disease by means of immunohistochemistry (Walsh et al., 2001), our immunohistochemical study showed an increase in intensity in almost all the assessed cytokines in scrapie affected sheep compared to healthy controls, supporting the role of cytokines in the pathogenesis of TSE.

In our results, a basal level of some markers in negative controls was shown. Effectively, it has been previously described that IL-6, for instance, is expressed constitutively in discrete regions of the CNS not only during illness but also under normal physiological conditions (Schobitz et al., 1993).

We had some problems with background in immunohistochemistry, the same as Parker and Smith, 1999 observed, confirming that every primary antibody they had used in cytokine detection had proved to be troublesome with regard to background. One possible explanation is that we have not used PLP fixation and low temperature paraffin-embedding, as it was described as ideal protocol for immunohistochemical detection of cytokines in mouse tissues (Whiteland et al., 1997).

Regarding the cellular location, previous studies have reported that some cytokine receptors are localized in granular layer but not in Purkinje cell layer in rat (Schobitz et al., 1993).In contrast, other authors have found that immunostaining in granular layer for such receptor was low and was higher in Purkinje cells in mouse (Nelson et al., 1999).This distribution is similar to that observed in our study: cytokine receptors are more expressed by Purkinje cells than by other cells in sheep cerebellum. The reason for these differences among studies may well be due to species-specificity.

As mentioned before, we considered of interest to determine the cytokine and cytokine receptors expression over scrapie progress in natural model. Previous studies regarding cytokines in models of prion diseases have not done this. Much of what is known about neuroinflammation in prion diseases has come from studies utilizing transgenic murine models. Our study adds growing research in the complex relationship of prion diseases with cytokines. However, the upregulation of cytokines requires further research efforts to determine its possible detrimental role in chronic neurodegeneration. The fact that cytokines play a key role in neuroinflammation and neurodegeneration has opened new areas of scientific investigation. Nonetheless, little is known about how chronic exposure of neurons to cytokines alters their function.

To elucidate whether significant differences regarding the behaviour of specific cells referred to inflammatory marker expression exist along the progress of the disease is the subsequent aim. Additionally, a further possibility could be considered: double staining experiments to elucidate whether the same cells are expressing the same cytokines. Either quantitative or molecular studies in order to stablish possible differences among distinct disease stages and identify specific cell type expressing these receptors have been initiated with the objective of elucidating consistent differences among distinct disease stages. In the future, we pretend to design a definitive neuroinflammatory profile in natural ovine scrapie.

**CONCLUSIONS**

Expression patterns of several cytokines have been assessed here, since they might be neuroinflammatory markers during natural scrapie progress. Immunosignalling corresponding to some neuroinflammatory cytokines has shown to be increased in affected animals compared to negative controls while it has been the contrary for others. These differences observed in terms of cell morphology, intensity and distribution pattern confirm that a complex network of cytokines is involved in the pathogenesis of natural scrapie.

This preliminary study shows an inflammatory profile in cerebellum belonging to scrapie affected sheep at different clinical stages of the disease. In the future, this study is planned to be extended to some other pro-inflammatory proteins in order to provide a global profile of the neuroinflammatory process in the group of prion diseases.

By means of identification of cellular type expressing these inflammatory markers, paying particular interest in astroglial and microglial populations as main components of the host

immune response in prion diseases, reliable conclusions about the process of neurodegeneration will be possible to be drawn. Undoubtedly, characterization of cytokine and cytokine receptors in glial cells will enable us to gain information of the bidirectional communication between the immune and the nervous system. Understanding cytokine action in scrapie could lead to novel therapeutic strategies, some of which are nowadays being used in clinical trials.

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# ESTUDIO Nº 2:

## ASSESSMENT OF HOST IMMUNE RESPONSE IN THE PROGRESS OF NATURAL SCRAPIE AFTER CHRONIC DEXAMETHASONE TREATMENT

# ESTUDIO Nº 2: ASSESSMENT OF HOST IMMUNE RESPONSE IN THE PROGRESS OF NATURAL SCRAPIE AFTER CHRONIC DEXAMETHASONE TREATMENT

**ABSTRACT**

Neuroinflammation has been correlated with the progress of neurodegeneration in many neuropathologies. Although glial cells have traditionally been considered to be protective, the concept of them as neurotoxic cells has recently emerged. Thus, a major unsolved question is the exact role of astroglia and microglia in neurodegenerative disorders. On the other hand, it is well known that glucocorticoids are the first choice to regulate inflammation and, consequently, neuroglial inflammatory activity.

The objective of this study was to determine how chronic dexamethasone treatment influences the host immune response to characterize the beneficial or detrimental role of glial cells. To date, this has not been examined using a natural neurodegenerative model of scrapie. With this aim, immunohistochemical expression of glial markers, prion protein accumulation, histopathological lesions and clinical evolution were compared to those in a control group.

The results provided here demonstrate how the complex interaction between glial populations fails to compensate for brain damage in natural conditions, emphasizing the need of using natural models. Additionally, the data show that modulation of neuroinflammation by anti-inflammatory drugs may become a research focus as a potential therapeutic target for prion diseases, similar to that considered previously for other neurodegenerative disorders classified as prion-*like* diseases.

**Keywords,** scrapie, dexamethasone, neuroinflammation, astrocytes, microglia, prion diseases.

**INTRODUCTION**

Prion diseases are a group of fatal neurodegenerative diseases affecting animal and human species caused by the conversion of cellular prion protein ($PrP^{c}$) into a pathological isoform, called pathological prion protein ($PrP^{sc}$). Specifically, scrapie is the archetype of all these disorders. In spite of the importance of this group of diseases on public health and despite being an endemic disorder in many countries worldwide, studies have mostly focused on experimental instead of natural models (van Keulen et al., 2000; González et al., 2005; Raymond et al., 2007; Tabouret et al., 2010). However, evidence of failure in reliability, mainly in Neuroscience research, is increasingly being published (Aisen et al., 2003; Gordon et al., 2007). Therefore, there is growing interest in studying natural models, especially of neurodegenerative disorders, such as prion diseases.

Neuropathological hallmarks of prion diseases include spongiform changes, vacuolation, astrogliosis, microglial activation, neuronal loss and accumulation of $PrP^{sc}$ (Barcikowska et al., 1993; Liberski and Ironside, 2004; Ironside et al., 2005). Moreover, several lines of research have found that this group of diseases shares pathological features with other human neurodegenerative disorders, such as the extracellular deposit of insoluble plaques of an aberrant protein, astrocytosis and microglial activation (DeArmond, 1993; Soto et al., 2003). Thus, they have been called prion-*like* disorders (Prusiner, 2013), which include Alzheimer's (AD), Parkinson's (PD) or Huntington's (HD) diseases, among others. In line with this fact, some studies have proposed using prion diseases as acceptable models to better understand the pathogenesis of prion-*like* disorders (Frost and Diamond, 2010; Duyckaerts et al., 2019).

Currently, neuroinflammation is considered an intrinsic characteristic of all mentioned neuropathologies (Ransohoff, 2016). Indeed, the first description of an innate inflammatory response in a neurodegenerative process was made 25 years ago in AD (Akiyama, 1994),and subsequent studies have also found inflammatory components in other prion-*like* (Aguzzi and Falsig, 2012) as well as prion diseases (Xie et al., 2013; Carroll et al., 2016; Iaccarino et al., 2017). Neuroinflammation has been correlated with the progress of neurodegeneration (Ransohoff, 2016), even being proposed as a pathogenic mechanism in disorders, such as AD (Heneka et al., 2014), supporting the hypothesis that neuroglia constitute potential neurotoxic cell populations (Williams, 1994a; Liberski et al., 1995; Kordek et al., 1996; Williams et al., 1997a; Riemer et al., 2000; Marella and Chabry, 2004; Gómez-Nicola et al., 2013). The neuroinflammatory process is defined as the prolonged activation of microglial cells with the consequent production of pro-inflammatory cytokines (Schwartz and Deczkowska, 2016). Moreover, despite the fact that this glial cell type has gained more attention than astrocytes in

this process, astroglia have also been demonstrated to be highly involved in scrapie (Chesebro et al, 2005; Sarasa et al, 2012; Hernández et al, 2014; Hollister et al, 2015), human prion (Victoria et al, 2016; Monzón et al, 2018; Carroll and Chesebro, 2019) and prion-*like* diseases (Pekny and Pekna, 2016; Liu et al, 2017; Garcés et al, 2019).

Nevertheless, the notion of neuroinflammation being a protective response against neurodegeneration is also supported by other authors. The inflammatory process appearing in several neurodegenerative diseases has been attributed to a protective role for both astroglia (Guitart et al., 2016) and microglia (Hanisch and Kettenman, 2007; Falsig et al., 2008; Zhu et al., 2016; Carroll et al., 2018). Therefore, a major unanswered question is what exact role glial cells (which constitute innate immune cells in the central nervous system, CNS) play in neurodegenerative disorders.

Synthetic glucocorticoids (GC) have been used therapeutically in several inflammatory disorders. Consequently, they would be the first choice to control neuroinflammation. In fact, these synthetic hormones have been demonstrated to represent the main regulators of neuroglial inflammatory activity (Sierra et al., 2008), resulting in clinical benefit in AD (Beeri et al., 2012). In the same manner as for AD or PD, modulation or inhibition of neuroinflammation might be a therapeutic target for prion diseases (Agguzi and Zhu, 2017).To our knowledge, some studies based on corticosteroid treatments were tested in scrapie many years ago, but the main focus was on targeting $PrP^{sc}$ or $PrP^{c}$ (Kimberlin and Walker, 1979, 1983; White et al., 2003) and not the neuroinflammatory process.

The specific objective of this study was to assess the effect of the synthetic GC dexamethasone (DEX) on the spread of scrapie in a natural sheep model, paying special attention to the differential expression of astroglial and microglial markers as main components of the host immune response in the brain.

The overall goal consisted of determining the beneficial or detrimental role of these glial cells in the neurodegenerative progress of prion diseases. Moreover, since the natural model of scrapie (archetype of prion diseases) was used here, it might be a feasible and reliable model to extrapolate results to prion-*like* diseases.

**MATERIALS AND METHODS**

All the following experimental procedures were previously approved by the Ethical Committee of University of Zaragoza (Reference number, PI41/16). All efforts were made to minimize animal suffering during the experiments and to reduce the number of animals used.

The experiments were performed on 10 healthy and 15 clinical scrapie Rasa Aragonesa ewes. Affected animals belonged to positive flocks of scrapie in Zaragoza (Spain), presented clinical signs and their status was confirmed by the presence of pathological prion protein in recto-anal mucosa associated lymphoid tissue biopsies (RAMALT, Figure 1). Healthy sheep belonged to negative flocks where no scrapie cases had been ever detected and absence of pathological prion protein by RAMALT biopsies as well as absence of clinical signs were confirmed. Their age ranged from 4 to 10 years and genotypes presented were all ARQ/ARQ except for one ARQ/ARH. All of them were housed in two independent groups (control and clinical) in aired and illuminated rooms with free access to daily concentrate plus food and water.

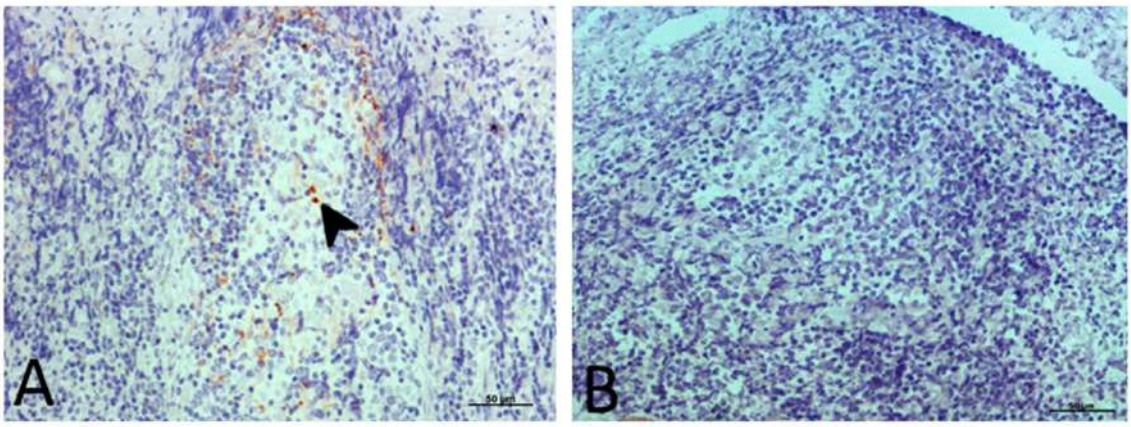


**Figure 1.** Immunostaining against pathological prion protein ($PrP^{sc}$, arrowhead) with L42 antibody in biopsies of recto-anal mucosa-associated lymphoid tissue (RAMALT). (**A)** Scrapie affected animal. (**B**) Control animal.

The clinical diagnosis of scrapie animals for inclusion in the study was based on classical signs such as pruritus, tremor, locomotor incoordination and behavioral changes. Healthy sheep included in the study evidenced absence of clinical signs before inclusion and throughout the experiment.

It has been considered crucial to include control animals in order to assess the effect of DEX treatment on healthy individuals because it had been not previously tested in ovine species. Of the total of animals included in the present study, 4 out of 10 controls and 10 out of 15 scrapie affected sheep at clinical stage were treated, respectively. A summary of cases is shown in Table 1.

**Table 1.** Summary of data corresponding to animals included in the study.

| Sheep Nº | *PRNP* genotype | Age (years) | Group | Treatment and Duration |
|---|---|---|---|---|
| **1** | ARQ/ARQ | 9 | Control | Untreated (13 months) |
| **2** | ARQ/ARH | 10 | Control | Untreated (16 months) |
| **3** | ARQ/ARQ | 7 | Control | Untreated (17 months) |
| **4** | ARQ/ARQ | 4 | Control | Untreated (17 months) |
| **5** | ARQ/ARQ | 4 | Control | Untreated (16 months) |
| **6** | ARQ/ARQ | 8 | Control | Untreated (9,5 months) |
| **7** | ARQ/ARQ | 8 | Control | Treated (10 months) |
| **8** | ARQ/ARQ | 5 | Control | Treated (16 months) |
| **9** | ARQ/ARQ | 8 | Control | Treated (16 months) |
| **10** | ARQ/ARQ | 4 | Control | Treated (16 months) |
| **11** | ARQ/ARQ | 9 | Clinical | Untreated (2 months) |
| **12** | ARQ/ARQ | 4 | Clinical | Untreated (<1 month) |
| **13** | ARQ/ARQ | 5 | Clinical | Untreated (1.5 months) |
| **14** | ARQ/ARQ | 4 | Clinical | Untreated (<1 month) |
| **15** | ARQ/ARQ | 5 | Clinical | Untreated (2.5 months) |
| **16** | ARQ/ARQ | 6 | Clinical | Treated (<1 month) |
| **17** | ARQ/ARQ | 4 | Clinical | Treated (<1 month) |
| **18** | ARQ/ARQ | 4 | Clinical | Treated (<1 month) |
| **19** | ARQ/ARQ | 4 | Clinical | Treated (<1 month) |
| **20** | ARQ/ARQ | 5 | Clinical | Treated (1 month) |
| **21** | ARQ/ARQ | 4 | Clinical | Treated (2 months) |
| **22** | ARQ/ARQ | 7 | Clinical | Treated (2 months) |
| **23** | ARQ/ARQ | 4 | Clinical | Treated (2 months) |
| **24** | ARQ/ARQ | 4 | Clinical | Treated (2 months) |
| **25** | ARQ/ARQ | 4 | Clinical | Treated (5 months) |

Treated sheep (control and clinical), were daily intramuscularly injected with DEX (SYVA, León, Spain; 0.04 mg/kg) in alternating posterior limb after a one-week period of acclimation and until euthanasia by endpoint criteria (16 months in the longest case). Non-treated animals

(control and clinical) were injected in the same place and conditions but with saline solution (SSF) and endpoint in untreated controls was established when experiment finished, since all animals of this group (but two, due to a second pathology) were alive. In addition, a daily dose of 0.5 mg/kg of omeprazole was administered to all sheep in order to avoid the appearance of gastric ulcers that GC can cause (Hoes et al., 2009). Clinical animals were monitored once per day for the development of clinical signs of the disease, which typically included behavioral changes (fixed stare, isolation, hyper-excitability), trembling, weight loss or emaciation, pruritus (main symptom in sheep, often leading to wool loss) and impaired vision (Dickinson, 1976; Hadlow et al., 1982; Bellworthy et al., 2008). Appearance of clinical signs unavoidably leaded to prostration which was defined as a humane end-point requiring euthanasia in both treated and non-treated clinical groups**.**

As expected, DEX treated sheep (mainly from control group, due to the extended duration of treatment) experienced lesions compatible with secondary effects of GC, including Cushing's syndrome. Nevertheless, wound lesions were the major safety concern due to long term use of DEX (3 of 4 control treated sheep affected by extensive alopecia). Due to this fact, they had to be euthanized by endpoint criteria.

Following intravenous pentobarbital injection and exsanguination, necropsy of each animal was performed. A total of 80 samples was collected and distributed for different studies. One hemi-section from each sample was fixed by immersion in 4% paraformaldehyde for histopathological and immunohistochemical studies and the other hemi-section was frozen at -80ºC for RT-qPCR studies. *Postmortem* interval between death and tissue processing was not lesser than 1 hour.

*HISTOPATHOLOGICAL STUDIES (H-E)*

Haematoxylin - eosin (H-E) staining was applied on paraffin-embedded 4 µm sections in order to visualize the neuropathological lesions in different brain areas, medulla oblongata (MO), obex (O), cerebellum (Cb) and frontal cortex (Fc). Spongiosis was assessed by count of the number of vacuoles present in the grey matter from each section and scored from 0 (minimum) to 4 (maximum) by two independent observers, as previously published by the group (Monzón et al., 2018; Garcés et al., 2019).

*IMMUNOHISTOCHEMICAL (IHC) TECHNIQUES*

Immunohistochemistry was carried out to assess the accumulation of $PrP^{sc}$, astrogliosis and microglial activation in the different brain areas selected.

After specific pre-treatments for antigen retrieval, immunohistochemical protocols by using specific primary antibodies against $PrP^{sc}$ and glial markers were applied. EnVision system (DAKO, Glostrup, Denmark) and diaminobenzidine (DAB; DAKO, Glostrup, Denmark) were used

as the visualization system and chromogen, respectively. Haematoxyllin counterstaining and mounting in DPX was performed on all sections. Table 2 summarizes all primary antibodies and protocols used.

**Table 2.** Primary specific antibodies used for immunohistochemical techniques and retrieval treatment applied for each antibody.

| Antibody | Antigen | Type | Dilution | Retrieval Method | Source |
|---|---|---|---|---|---|
| L42 | $PrP^{sc}$ | Monoclonal | 1:500 | Formic acid, 15 min<br>Proteinase K, 15 min<br>Heat treatment, 20 min<br>Peroxidase blocking | DAKO |
| Anti-GFAP | GFAP | Polyclonal | 1:500 | Peroxidase blocking | DAKO |
| Anti-IBA-1 | IBA-1 | Polyclonal | 1:1000 | Heat treatment, 20 min<br>Peroxidase blocking | WAKO |

As previously described (Monzón et al., 2018; Garcés et al., 2019), all slides were assessed by two independent operators who scored the intensity of $PrP^{sc}$ accumulation and gliosis from 0 (absence) to 4 (maximum). Glial morphology in 10 microscopic fields in each brain region was also evaluated.

- **$PrP^{sc}$ detection**

As previously published (Monleón et al., 2004),98% formic acid immersion for 15 min, proteinase K (4 µg/ml; Roche, Reinach, Switzerland) treatment for 15 min at 37ºC and hydrated heating for 20 min preceded the endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min and incubation with monoclonal antibody L42 (1,500, 30 min RT; DAKO, Glostrup, Denmark).

- **Glial Fibrillary Acidic Protein (GFAP) detection for astrogliosis**

After endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min, slides were incubated with a polyclonal antibody against glial fibrillary acidic protein (GFAP, 1,500, 30 min RT; DAKO, Glostrup, Denmark).

- **Ionized Calcium-Binding Adaptor Molecule-1 (IBA-1) detection for microgliosis**

Heat treatment during 20 min was necessary before endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min. Afterwards, sections were incubated with a polyclonal antibody against ionized calcium binding adaptor molecule 1 (IBA-1, also known as Allograft Inflammatory Factor 1, AIF-1, at 1:1.000; overnight 4ºC; WAKO, USA).

*RT-qPCR*

Cerebellum and frontal cortex frozen tissues from treated and non-treated clinical sheep were included in the following comparative molecular analysis for some glial markers.

- **RNA purification**

RNA purification was performed following supplier's instructions (RNeasy Lipid Tissue Mini kit, Qiagen, GmbH, Hilden, Germany). RNA integrity and 28S/18S ratios were determined with the Agilent Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA). Samples were treated with DNase digestion, and RNA concentration was evaluated using a NanoDrop Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Only RNA samples with OD 260/280 ratios close to 5.0 were selected for reverse transcription.

- **Retro-transcription**

RNA retro-transcription into cDNA was performed according to the manufacturer's manual (High-Capacity CDNA Reverse Transcription Kit, Applied Byosistems).

- **RT-qPCR**

Gene expression of astroglial markers GFAP and Aldehyde dehydrogenase 1 family member L1 (ALDH1L1) was assessed. Similarly, gene expression of microglial markers Allograft Inflammatory Factor 1 (AIF1, also known as IBA-1) and CD68 molecule (CD68) was also assessed. The parameters of the reactions were as following, 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Table 3 shows TaqMan probes used for these studies. Data were assessed using the ΔΔCt method, using Hypoxanthine Phosphoribosyl transferase 1 (HRPT-1) and β-glucuronidase (GUS-β) as reference genes.

**Table 3.** TaqMan probes used for the RT-qPCR analysis.

| Gene | Full name | Reference | Source |
|---|---|---|---|
| **AIF-1** | Allograft inflammatory factor-1 | Oa03222904_g1 | Thermo Fisher |
| **ALDH1L1** | Aldehyde dehydrogenase 1 family member L1 | Oa03267152_m1 | Thermo Fisher |
| **CD68** | CD68 molecule | Oa04741636_g1 | Thermo Fisher |
| **GFAP** | Glial fibrillary acidic protein | Oa03251662_m1 | Thermo Fisher |
| **GUS-β** | β-Glucuronidase (reference gene) | Oa04828868_m1 | Thermo Fisher |
| **HPRT-1** | Hypoxanthine phosphoribosyl transferase-1 (reference gene) | Oa04825272_gH | Thermo Fisher |

*STATISTICAL ANALYSIS*

Kaplan-Meier survival curves of both treated and non-treated clinical animals were performed. Statistical differences between curves were evaluated with Log Rank (Mantel-Cox test).

For results provided by IHC techniques, the normality of distribution was first tested with the Kolmogórov-Smirnov test. The nonparametric Mann-Whitney *U* test was used to assess quantitative differences between treated and non-treated groups. Moreover, a multivariate lineal regression was performed in order to detect whether some differences for GFAP marker could be associated to treatment.

Data provided by RT-qPCR were evaluated by Student's *t* test after assessing normality with Kolmogórov-Smirnov test.

SPSS software (SPSS Statistics for Windows, Version 17.0) was used for all analyses and significance in all cases was considered at $*p < 0.05$. All graphs were performed with GraphPad Prism 6.0. Data presented in Figures are expressed as means and the standard error of the mean (mean +/- SEM).

All statistical analyses were advised and supervised by *Servicio de Apoyo Metodológico y Estadístico* (SAME) – *IACS.*

**RESULTS**

*CLINICAL SIGNS*

Clinical sheep (regardless of treatment or non-treatment) showed motor symptoms (tremors, ataxia, incoordination, prostration) likely associated with deep affectation of the cerebellum, as well as pruritus in most cases. Table 4 shows the main clinical signs developed by each clinical sheep during the experiment. Control sheep did not present any of the key features of scrapie throughout the time of the experiment in any cases.

**Table 4.** Main clinical signs related to scrapie developed by both dexamethasone (DEX-treated) and non-treated clinical sheep.

| Group | Sheep Nº | Main clinical signs |
|---|---|---|
| Clinical non-treated | 11 | Tremors, pruritus, ataxia, lost look |
| | 12 | Pruritus with skin lesions, alopecia, hyper excitation |
| | 13 | Pruritus, alopecia, prostration |
| | 14 | Tremors, alopecia, prostration |
| | 15 | Pruritus |
| Clinical DEX-treated | 16 | Pruritus, ataxia, tremors |
| | 17 | Pruritus, alopecia |
| | 18 | Tremors, ataxia, hyper excitation |
| | 19 | Pruritus, alopecia, hyper excitation |
| | 20 | Tremors, intense widespread alopecia, prostration |
| | 21 | Hyper excitation, prostration |
| | 22 | Tremors, constant pruritus, bruxism |
| | 23 | Scarce pruritus |
| | 24 | Intense pruritus with skin lesions |
| | 25 | Pruritus, alopecia, prostration |

As expected, DEX-treated sheep (mainly from the control group, due to the extended duration of treatment) experienced lesions compatible with secondary effects of GC, including Cushing's syndrome in 3 of 4 cases. Nevertheless, wound lesions were the major safety concern due to long term use of DEX (3 of 4 control-treated sheep were affected by extensive alopecia).

Regarding survival time, although no statistically significant differences were observed between Kaplan-Meier curves in clinical sheep, one of the ten (10%) treated clinical sheep survived 155 days compared to 72 days as a maximum for the non-treated clinical animals (Figure 2).

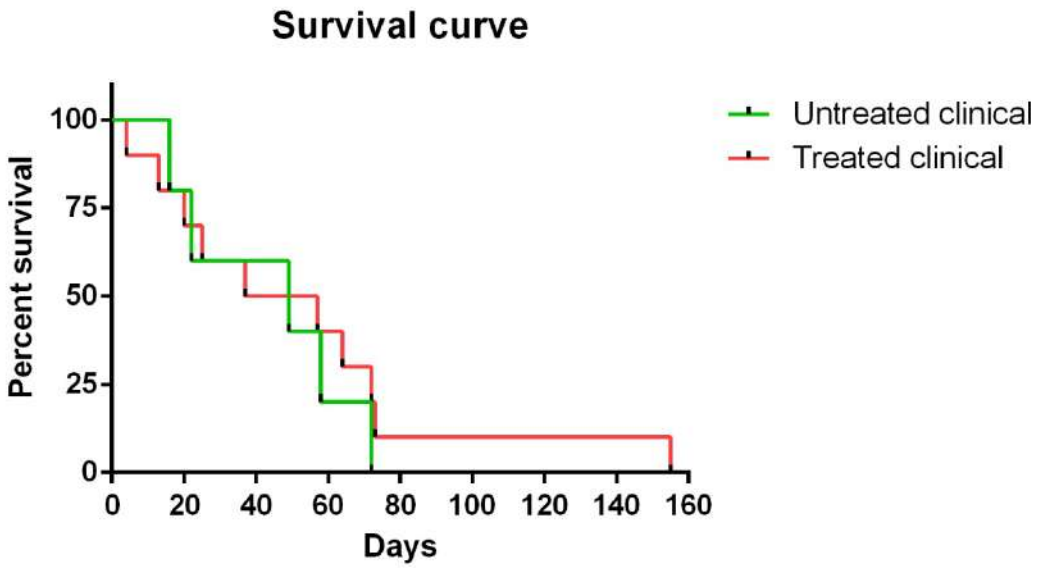


**Figure 2.** Kaplan–Meier survival curves corresponding to non-treated and treated clinical sheep. Note that one of ten treated clinical sheep survived 155 days, which was much longer than the maximum (72 days) observed for non-treated clinical animals.

*HISTOPATHOLOGICAL FINDINGS (H-E)*

Spongiosis was absent in all regions examined in sheep samples of both treated and non-treated control groups, while it was widespread in all areas in the clinical groups. Vacuolation was mainly located in the neuropil in all cases where it was found, although intraneuronal vacuoles were also observed in some cases. In regard to brain regions, subtle spongiform changes were found in Fc, while they were much more pronounced and severe in MO from all clinical animals. Thus, caudal tissues were the most affected by spongiform changes (Figure 3A). Regarding the specific impact of DEX administration on spongiform change, no significant differences were found between treated and non-treated clinical sheep ($p > 0.05$) (Figure 3B).

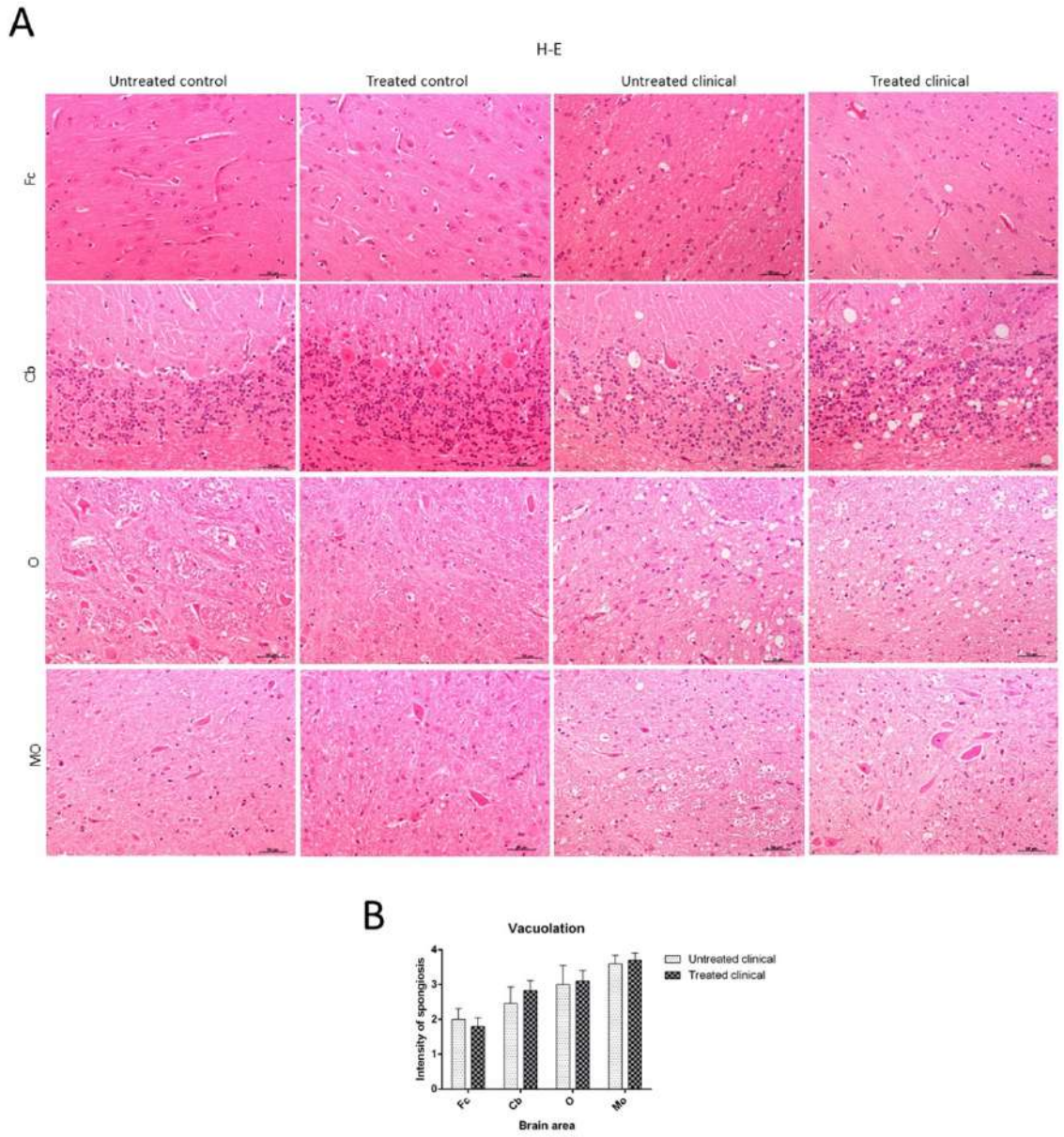


**Figure 3.** Vacuolation intensity (H-E staining). (**A**) Spongiosis was absent in all regions examined in both treated and non-treated control groups, while it was widespread in all tissues in clinical groups. Vacuolation was most frequently located in the neuropil. Note that spongiform change was most pronounced and severe in most caudal tissues. (**B**) Statistical analysis evidenced no differences between treated and non-treated clinical sheep. Medulla oblongata (MO), obex (O), cerebellum (Cb) and frontal cortex (Fc).

The significant decrease in motor activity observed in treated and non-treated clinical sheep was consistent with cell damage observed in the Purkinje cell layer, which presented severe vacuolation and even partial disappearance of Purkinje cells in some cases. Furthermore, these cells appeared swollen and with neurite thickening (torpedoes) (Figure 4).

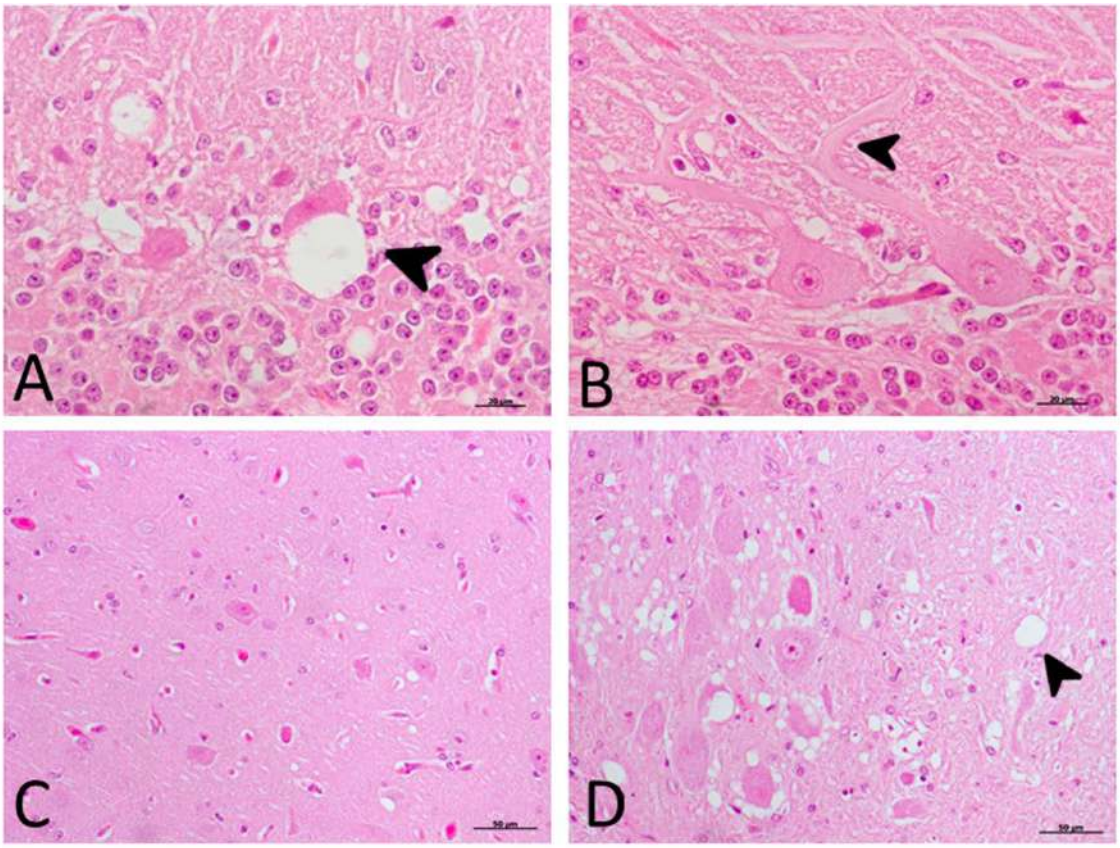


**Figure 4.** Main morphological findings in clinical sheep (H-E staining). **(A).** Cell damage observed in Purkinje cell layer of cerebellum. Note the severe intraneuronal vacuolation (arrowhead) and partial disappearance of Purkinje neurons**. (B)** Purkinje cells appeared swollen and with higher neurite thickening (torpedoes, arrowhead). **(C)** Subtle spongiform change was found in the frontal cortex. **(D).** Meanwhile, neuropil vacuolation (arrowhead) was found pronounced and severe in the medulla oblongata in all cases.

*IMMUNOHISTOCHEMICAL FINDINGS (IHC)*

- *$PrP^{sc}$ accumulation*

No $PrP^{sc}$ deposits were observed in any tissues belonging to control animals. On the other hand, prion protein deposition was widespread in all brain areas examined in both the treated and non-treated clinical groups. Quantitative differences concerning $PrP^{sc}$ deposits were found between Cb and Fc in both groups of clinical animals (Figure 5A). No significant differences between treated and non-treated clinical animals were found by *U*-Mann-Whitney test (Figure 5B).

Although lineal, spot, coalescent or granular $PrP^{sc}$ deposition patterns could be observed on some occasions, the coalescent pattern was the most frequently observed in all brain regions. Purkinje cells in the cerebellum were never immunostained for this marker.

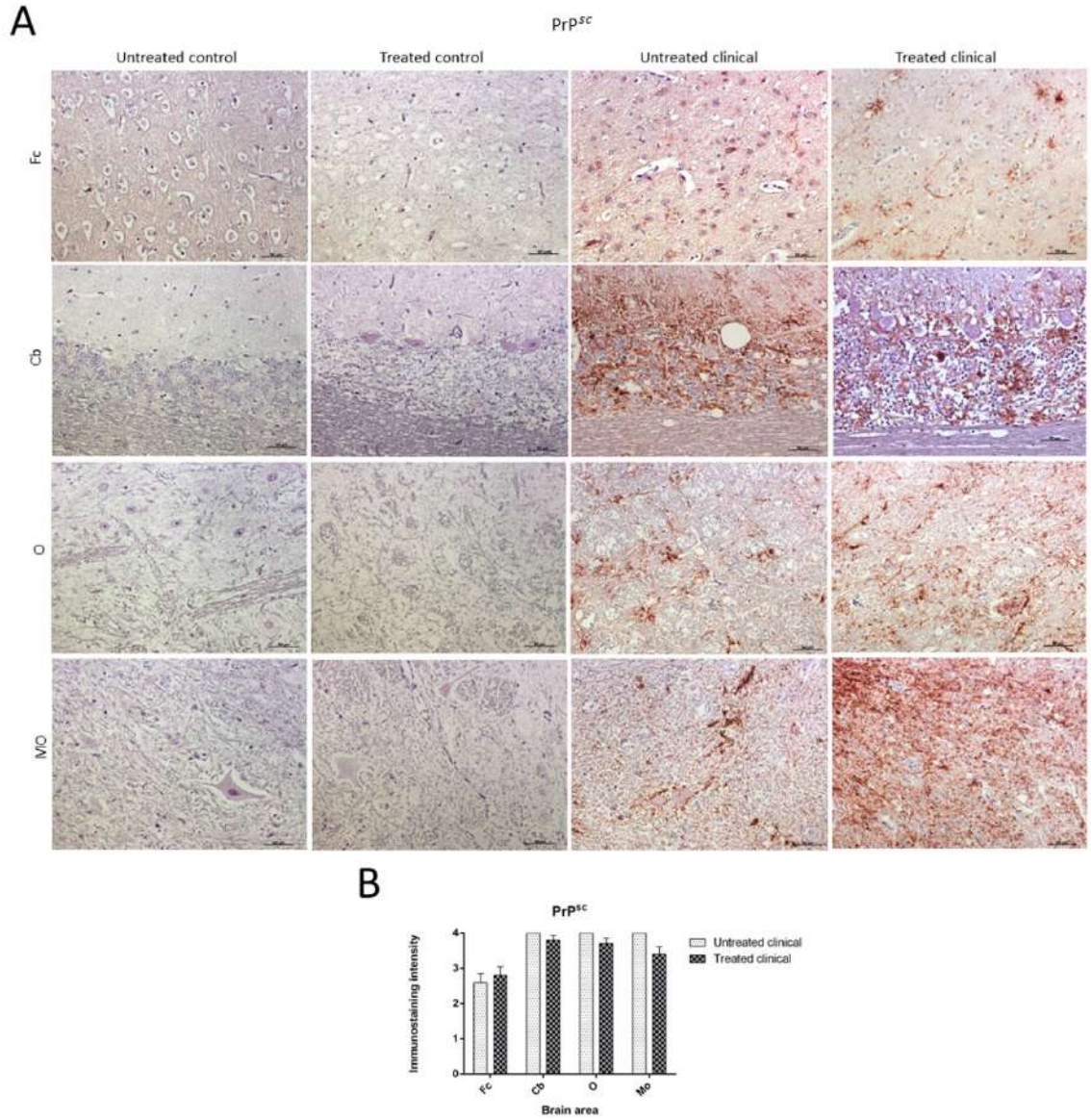


**Figure 5.** Pathological prion protein deposition by immunohistochemistry (IHC) with L42. (**A)** No $PrP^{SC}$ deposits were observed in any tissues belonging to animals from control groups. $PrP^{SC}$ deposition was widespread in all brain regions examined in both treated and non-treated clinical groups. (**B)** No statistical differences between treated and non-treated clinical sheep were found. Medulla oblongata (MO), obex (O), cerebellum (Cb) and frontal cortex (Fc).

- *GFAP*

As previously described, paraffin sections processed for IHC showed an increase in GFAP immunoreactivity in clinical scrapie when compared with controls (Figure 6A).

Treated control animals exhibited significantly higher immunolabelling for GFAP than the respective non-treated group in all regions but the obex (Fc * $p < 0.05$, Cb * $p < 0.05$ and MO ** $p < 0.01$) (Figure 6B). Strikingly, by contrast, GFAP immunostaining did not reveal major differences regarding an effect of treatment in any brain area examined in both clinical groups

(Figure 6C). Multivariate linear regression analysis verified that the effect of treatment was significant ($p < 0.05$).

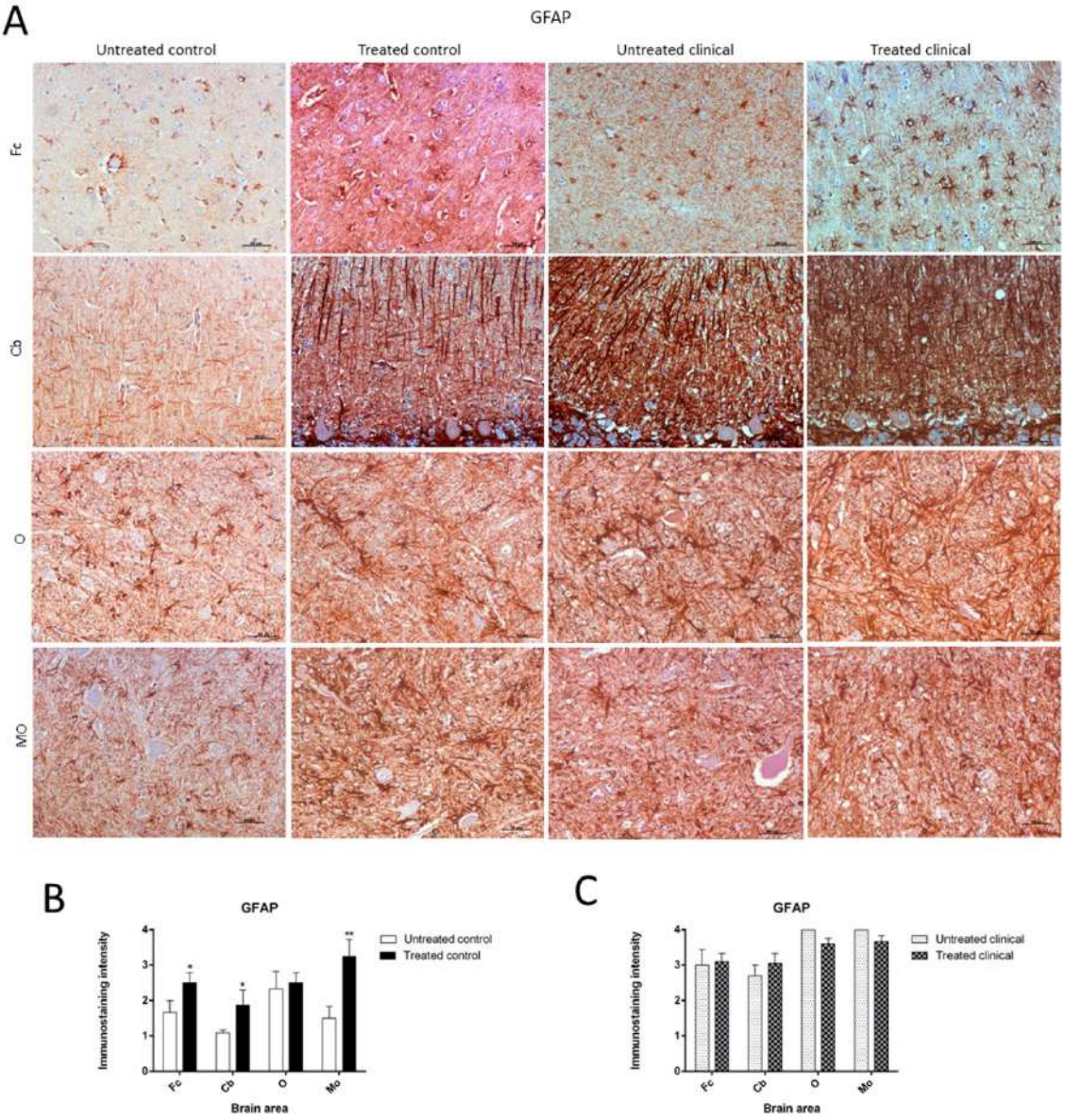


**Figure 6.** Glial fibrillary acidic protein (GFAP) immunostaining. (**A**) Increase of GFAP immunoreactivity in non-treated clinical scrapie compared with non-treated controls was evidenced. (**B**) Treated control animals showed significantly higher immunolabeling for GFAP than non-treated animals in all regions, except the obex. (**C**) GFAP immunostaining in the four regions examined did not reveal differences between treated and non-treated clinical animals. Medulla oblongata (MO), obex (O), cerebellum (Cb) and frontal cortex (Fc).

Morphologically, GFAP immunolabelling was found surrounding the meningeal zones in all regions examined. In the cerebellum, as previously described, the Purkinje cell layer was often immunostained, and an intense radial profile of GFAP in the molecular layer was found in samples with the highest intensity. Meanwhile, samples with the lowest GFAP intensity

predominantly presented a horizontal profile in this layer of the cerebellum. In addition, the vast majority of astrocytes in the medulla oblongata and obex showed hypertrophic morphology in treated controls compared to astrocytes in the non-treated sheep, which demonstrated their typical stellate morphology (Figure 7).

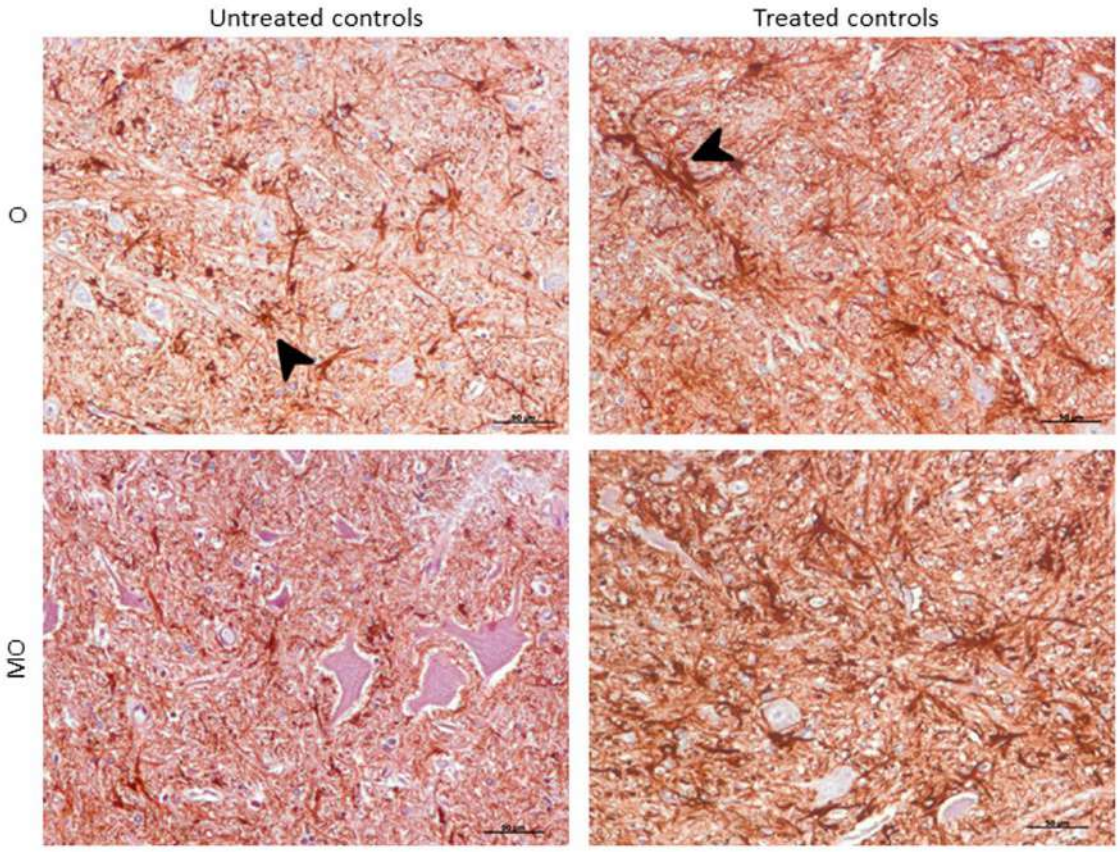


**Figure 7.** Morphological findings by GFAP IHC. Astrocytes in the medulla oblongata (MO) and obex (O) were widespread hypertrophic (arrowhead) after DEX treatment in controls compared to astrocytes in the non-treated ones, which appeared to be ramified (arrowhead).

- *IBA-1*

As expected, the IHC technique demonstrated an expansion of microglial populations (IBA-1 + cells) in clinical animals when compared with controls. However, no differences were found regarding the intensity of microgliosis among brain regions either for the control or clinical groups (Figure 8A).

Contrary to those observed changes for astroglia in controls, the microglia immunostaining pattern did not significantly change upon DEX treatment during clinical or control stages. Thus, no statistically significant differences in intensity were found between microglia in animals with or without treatment (Figures 8B, 8C).

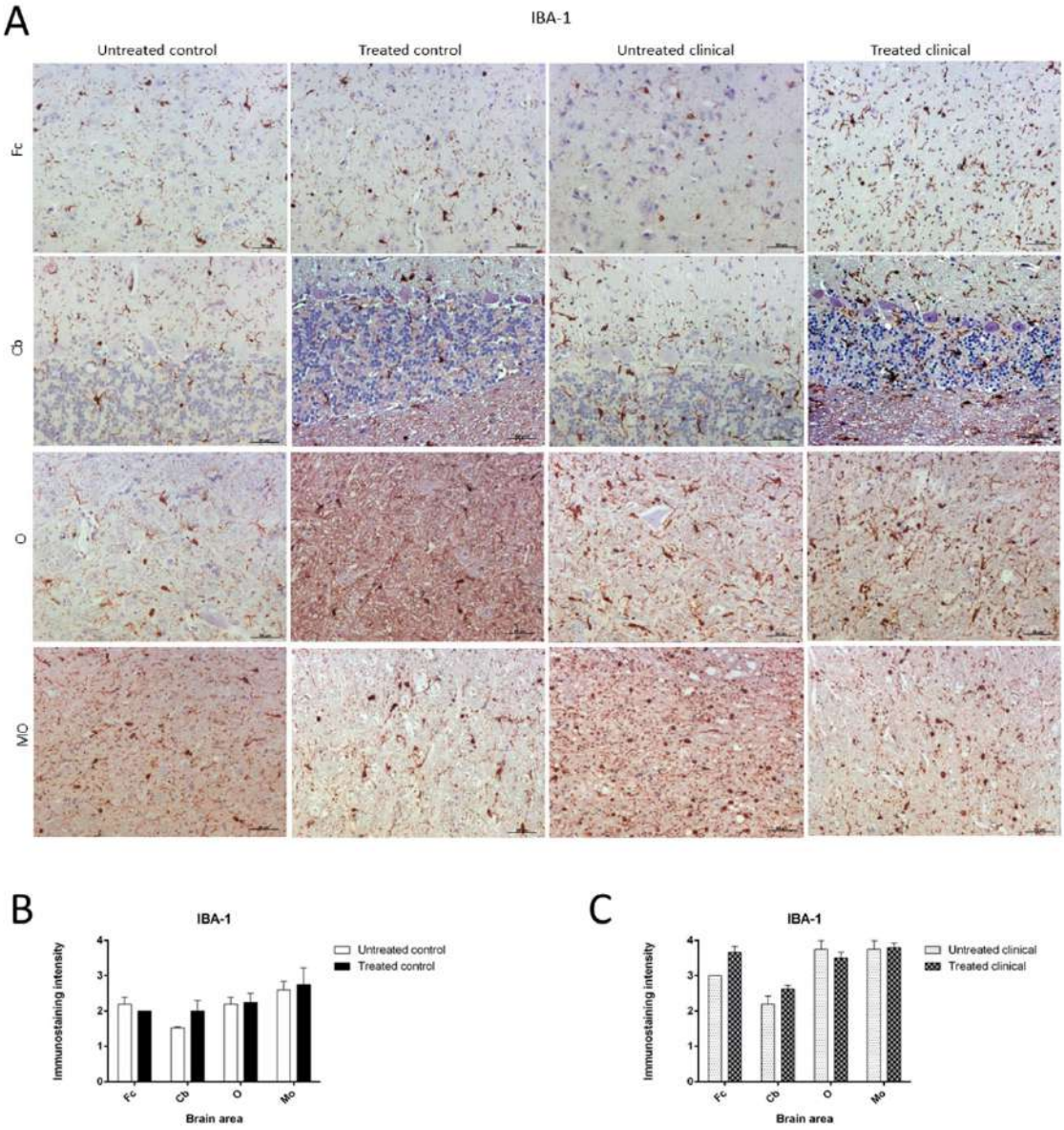


**Figure 8.** Ionized calcium-binding adaptor molecule-1 (IBA-1) immunostaining. (**A**) Microglial immunostaining intensity did not change with DEX treatment in the control and clinical groups. (**B, C**) No statistically significant differences in IBA-1 intensity between treated and non-treated animals were found. Medulla oblongata (MO), obex (O), cerebellum (Cb) and frontal cortex (Fc).

Morphologically, the Purkinje cell layer in the cerebellum was never immunostained for IBA-1, and the ramified phenotype was the most frequently observed morphology. Additionally, a relevant higher percentage of microglial cells in this encephalic area presented an amoeboid phenotype in treated controls compared to microglia in the non-treated sheep (Figure 9).

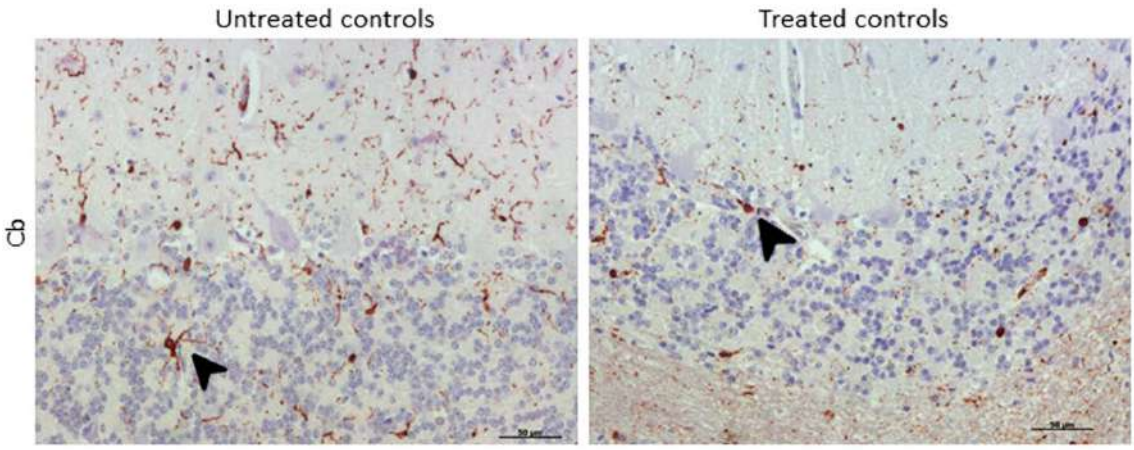


**Figure 9.** Morphological findings by IBA-1 IHC. A higher proportion of microglial cells in cerebellum presented an amoeboid (arrowhead) phenotype in DEX-treated controls compared to that in non-treated ones, which appeared to be mostly ramified (arrowhead).

*RT-qPCR*

Although it was not statistically significant ($p$ = 0.064), daily injections of DEX in clinical sheep tended to show increased GFAP mRNA levels in the cerebellum in comparison with non-treated clinical animals. However, no significant differences were found in the mRNA expression of the microglial markers tested (AIF1 and CD68 genes) in the cerebellum (Figure 10A).

Meanwhile, regarding the frontal cortex, AIF1 (or IBA-1) expression tended to be lower in clinical DEX-treated sheep compared to the non-treated group, although this was not statistically significant ($p$ = 0.085). No changes were detected for astrocytic markers (GFAP and ALDH1L1) in this brain region (Figure 10B).

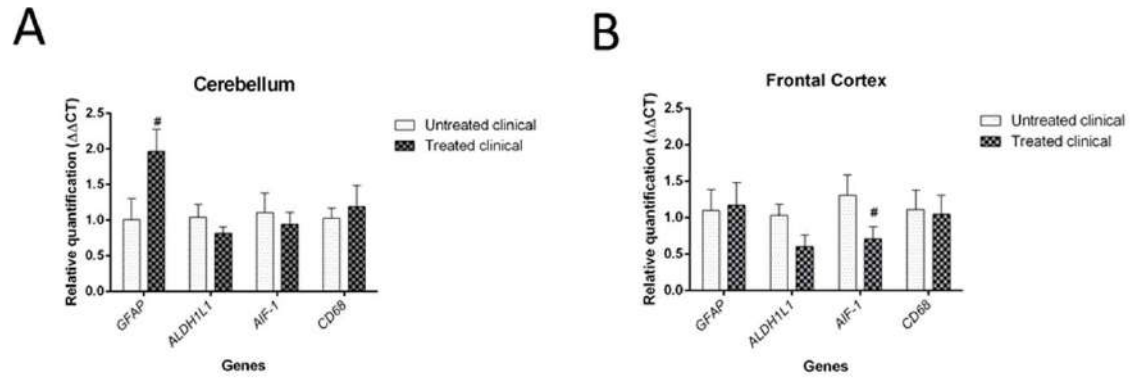


**Figure 10.** Results of gene expression by RT-qPCR in clinical individuals. **(A)** DEX treatment evidenced a tendency (#) toward increased GFAP mRNA levels in the cerebellum of treated animals compared with non-treated animals, although it did not reach statistical significance ($p$ = 0.064). (**B)**. Although it was no statistically significant ($p$ = 0.085), there was a tendency toward decreased of AIF-1 gene expression (microglia) in the frontal cortex of treated animals compared with that of animals in the non-treated group.

**DISCUSSION**

Currently, no treatments for prion diseases exist. To our knowledge, most of the few studies testing potential treatments in scrapie as an archetype of this group of disorders were carried out many years ago. Moreover, their main focus was on targeting $PrP^{sc}$ or $PrP^{c}$ instead of the process of neuroinflammation. Here, the effect of long-term GC administration on survival period, $PrP^{sc}$ deposition, spongiosis and glial response was evaluated using naturally infected scrapie sheep as a natural model of neurodegenerative disease. Thus, the purpose of this experiment was to determine how the synthetic GC DEX influences the host immune response in order to characterize the beneficial or detrimental role of glial cells in the progress of neurodegeneration of prion diseases. Previous studies testing different strategies to manipulate microglial function reported contrary effects on survival period in experimental models of prion disease (Gómez-Nicola et al., 2013; Carroll et al., 2018). This prompted us to more deeply investigate the role of host immunity during the course of natural infection.

Intensive research efforts have been spent looking for a therapeutic solution for neurodegenerative diseases based on immunomodulation. The development of therapeutic strategies that inhibit NF-kB activity was proposed to tackle these pathologies (Grilli and Memo, 1999). In fact, DEX has been used as a glio-modulator candidate (Efremova et al., 2016) and was found to protect neurons via decreased neuroinflammation of glial cells in a mouse model of PD (Kurkowska-Jastrzebska et al., 2004). More recently, GC therapy has been demonstrated to present clinical benefit in AD (Beeri et al., 2012) and is associated with a lower risk of dementia in humans (Nerius et al., 2020). In contrast, GC has been described as neurotoxic for AD in murine models since they enhance the pathology, augmenting deposits of amyloid beta and tau (Green et al., 2006). In regard to prion diseases, Outram et al., 1974 observed a reduction in the susceptibility to murine scrapie infection of mice treated with GC. Treatment with ibuprofen in murine scrapie was not conclusive due to the risky side effects (Riemer et al., 2008).

The results described here regarding scrapie at clinical stages of disease partially agree with previous reports showing that no treatment is effective when neuronal degeneration has already begun. This is consistent with descriptions for non-steroidal anti-inflammatory drugs (NSAIDs) in AD (Imbimbo, 2009), suggesting that prevention strategies are necessary instead of treatments. No beneficial long-term effect on disease progression was demonstrated for GC therapy in multiple sclerosis (MS) (Tischner and Reichardt, 2007). However, it is worth mentioning that, in the present study, one clinical sheep extended its survival period after DEX treatment. Even though this was only observed for one individual, it represents an

encouraging result, as it opens hope for anti-inflammatory therapy to have potential in at least some cases. Further studies to understand this potentiality are being designed.

Of note, corticosteroids have been established to cause dose-related immunosuppression, yet the mechanisms behind this impaired immune function have not been defined (Min et al., 2015).GC exhibit paradoxical immunomodulatory functions (Munck and Toth, 1992, Filaretova et al, 2009); although traditionally known as anti-inflammatory drugs, sometimes the modulating mechanisms fail, and they can aggravate CNS inflammation (Sorrells et al., 2009). The paradoxical effects of GCs on neuronal survival and death have been attributed to the concentration and the ratio of receptor activation. (GILZ) is a recently identified protein transcriptionally upregulated by GCs. Constitutively expressed in many tissues including the brain, GILZ mediates many of the actions of GCs. It mimics the anti-inflammatory and antiproliferative effects of GCs, but also exerts differential effects on stem cell differentiation and lineage development. Together with doses of GC, the length of treatment in relation to the immune response is decisive to determine if GC exhibits pro- or anti-inflammatory properties (Spies et al., 2011). Acquired resistance is another problem, according to descriptions in MS after treatment for 3-6 months (Kirwan, 2007). All these reasons could explain the lack of differences observed in this study regarding length of treatment, despite varying from some weeks to nearly 18 months.

Although some studies argue that DEX has minimal access to the CNS (Meijer et al, 1998; De Kloet et al, 1998; Hueston and Deak, 2014), the study presented here clearly demonstrates successful efficacy of DEX to cross the blood-brain barrier, as evidenced by the strong astroglial reaction in treated controls. However, this experiment also presented further complications due to the issue of GC therapy itself. DEX has a number of adverse effects, mostly associated with suppression of immunity (Giles et al., 2018). The same side effects as those described by other authors (Weindl et al, 2004; Whitehouse, 2011) arose here, with outstanding wound loss as the most frequent and highly adverse side effect. On many occasions, animals needed to be euthanized because of this effect. For this reason, it is difficult to conclude whether this immunosuppressive therapy might ameliorate clinical signs or slow down the neurodegenerative process. The provided data should be interpreted with caution due to the use of a natural model along with the inherent difficulties described above.

Regarding the specific impact of DEX administration on neuropathological lesions, no significant differences in vacuolation or $PrP^{sc}$ deposition were found between treated and non-treated clinical sheep. The observed cell damage in the Purkinje cell layer of the cerebellum is in agreement with previous findings in scrapie (Hernández et al., 2014),Creutzfeldt-Jakob disease (CJD) (Monzón et al., 2018) and other neurodegenerative disorders (Sarna and

Hawkes, 2003). When these cells were closely observed in ultrastructural studies, the vacuolation occurring around this cell type displayed a close relationship with glial cells (Sarasa et al., 2015). In regard to differences among brain regions, the most caudal areas were the most affected by spongiform changes and $PrP^{sc}$ deposits. They were more frequent and widespread in the cerebellum compared to the frontal cortex, as previously reported in scrapie (Lezmi et al., 2011). $PrP^{sc}$ presented different deposit patterns, although coalescent ones were the most frequently observed in all brain regions, as was previously found for the cerebellum from CJD-affected individuals (Monzón et al., 2018). In contrast, a recent study demonstrated that $PrP^{sc}$ accumulates in all brain regions independently of neurodegeneration (Alibhi et al., 2016). This discrepancy in results might be due to differences in models, since this last study used a mouse experimental model of Gerstmann-Sträusler-Scheinker, and the other researchers used natural models of scrapie and CJD. This reinforces that we should be cautious with extrapolation from experimental models to reality.

Focusing on glial cells, which are the key topic in this study, both activated astrocytes and microglia, based on morphology as well as specific marker expression, were observed in clinical animals compared to controls. Activation of glia in scrapie (Sarasa et al., 2012; Hernández et al., 2014) and other prion-*like* diseases (Akiyama et al., 2000; Garcés et al. 2019) has been exhaustively characterized. However, this has not previously been described as a result of anti-inflammatory treatment.

In this study, a statistically significant difference in GFAP marker expression was observed in all regions examined, except the obex, after DEX treatment compared to controls. By contrast, treatment did not reveal major differences between clinical animals. This may be due to downregulation of astrogliosis, reflecting astroglial paralysis in the clinical stage of scrapie, as was recently described in late stages of AD (Verkhratsky et al., 2015, 2019). Further studies would be essential to confirm this assertion.

The cerebellum is a preferential target of prions in scrapie (Fraser et al., 1996; Williams et al., 1997a) and CJD (Berciano et al., 1990; Armstrong et al., 2009; Cali et al., 2015). In fact, a relevant finding in this encephalic area could help to clarify astrocytic behaviour in the neurodegenerative progress of prion disease. An intense radial profile was observed in the molecular layer with higher intensity, while the horizontal profile was instead related to samples with low intensity of this marker. This is the same as that evidenced in our previous works for human prion and prion-*like* disease samples (Monzón et al., 2018; Garcés et al., 2019). As claimed then, this pattern suggested a possible glial stem cell response in order to protect against or compensate for neuronal loss (Álvarez et al., 2015).This would agree with the hypothesis about astroglial paralysis, which, despite trying to react against brain damage,

might prevent competent astrocytes from being formed. In the same vein, RT-qPCR results demonstrated a tendency towards increased GFAP mRNA, which is consistent with this assumption. Even though astroglia seem to attempt to compensate for the damage by initiating proliferation/regeneration, an error somewhere in this process could be blocking the final aim.

Another GFAP morphological finding was that the vast majority of astrocytes presented a hypertrophic morphology in treated control samples (similar to that at the clinical stage) in comparison with the typical stellate form in non-treated samples. It is well known that microglia undergo complex metamorphoses when they are reactive. However, an enigma of control of astroglial morphology in brain physiology is beginning to emerge (Zeug et al., 2018). Indeed, this finding is, to our knowledge, the first description of this phenomenon in astroglia.

In this *in vivo* study, comparing non-treated clinical and control samples, there was also an expansion of the microglial population in the clinical stage, as expected. This resulted in increased number of microglia associated with an activated and phagocytic phenotype, as previously reported (Perry et al., 2002; Perry and O'Connor, 2010). Nevertheless, microglial immunostaining intensity did not significantly change after DEX treatment (neither in the clinical or control group). Similarly, a previous study (Meneses et al., 2017) did not observe a decrease in microglial activation after LPS induction of neuroinflammation and intranasal DEX treatment in mice, suggesting that the dose was not sufficient to reduce IBA-1 expression. However, other studies managed to reduce microglia activation with DEX sodium phosphate by using cell cultures (Hui et al., 2020). The discrepancies in results may be due to different experimental models, pharmacological presentation or routes of DEX administration. Indeed, recent studies have shown that the effects of GC on brain inflammatory responses are truly complex (Duque Ede and Munhoz, 2016).

Concerning morphological findings, a high percentage of microglial cells in the cerebellum presented an amoeboid phenotype in treated controls compared to non-treated ones, which appeared more ramified. Provided that the amoeboid phenotype represents the most activated microglial shape (Ransohoff and Perry, 2009; Boche et al., 2013; Streit et al., 2014) associated with expression of neuroinflammatory genes (Kim and de Vellis, 2005), and the presence of this amoeboid phenotype in prion diseases seems to be stimulated by the high accumulation of $PrP^{sc}$ (Muhleisen et al., 1995), we might speculate that DEX treatment stimulates phagocytosis of $PrP^{sc}$ deposits, which would constitute a useful tool against prion progress. However, this stimulation is not evident in clinical animals despite the same treatment administration. This is consistent with the idea of a failure of the glial response.

Regarding gene expression analysis, DEX treatment in clinical sheep involved an increase of GFAP as well as a decrease in AIF1 (also known as IBA-1) mRNA expression in the cerebellum and frontal cortex, respectively. This finding could be related to the previously described region-specific pattern of neuroinflammation in sporadic CJD (Llorens et al., 2014), in accordance with different cytokine profiles found in these two brain regions. It is worth mentioning that glia immunostaining and its respective mRNA expression were not correlated in recently reported previous studies (Norden et al., 2016; Meneses et al., 2017), an aspect that needs further clarification. Nevertheless, in this study, the tendencies demonstrated by high resolution molecular analysis confirm the overall results provided by IHC analysis. Consequently, both techniques, which here were demonstrated to complement one another, lead to the conclusion of a probable glial failure.

It is currently assumed that glial responses can play both protector and toxic roles depending on the degree of activation (DiSabato et al., 2016; Obst et al., 2017; Carroll and Chesebro, 2019), supporting the concept that neuroinflammation induced by glia can amplify pathology (Liberski et al., 1995; Kordek et al., 1996). The transition from neuroprotective to neurotoxic activity of astrocytes by cytokine stimulation has been demonstrated (Buffo et al., 2010; Garwood et al., 2011; Efremova et al., 2016), but such neurotoxicity was prevented when astrocytes were treated with DEX in cell culture, while DEX had no effect on neurons (Debroas et al., 2015). On the basis of these observations, DEX could promote neuroprotective properties of astrocytes, as confirmed here in the natural model of prion disease. Nevertheless, the findings presented in this study support a potential failure of astrocytes and not a role for enhancing pathology.

A recent study reported that neurotoxic astrocytes (called A1) are potential contributors to neuronal death in several neurodegenerative disorders (Liddelow et al., 2017; Liddelow and Barres, 2017). This subtype of astrocytes might be involved in damaging actions, although the interaction of both populations, astroglia and microglia, has been postulated as a candidate involved in neurodegeneration (Liddelow et al., 2017; Kirkley et al., 2017), resulting in the essential presence of microglia (Kirkley et al., 2017). As a consequence, drugs to block the release of neurotoxins by these astrocytes have been suggested as a possible solution (Liddelow et al., 2017).

Currently, the activation of astrocytes remains poorly understood. The release of cytokines from those astrocytes and microglia accompanies this event. Provided that the present study is focused on glial cells, the next goal would consist of providing new information about the mechanisms underlying cytokine release. As has been previously suggested, it could be a

crucial target for therapeutic approaches in CNS in prion diseases (Garwood et al., 2011). It is indispensable to study how glial communication might prevent neuronal damage.

Distinct conformations of $PrP^{sc}$ might explain the unusual wide range of neuropathological, biochemical and clinical features of prion diseases (Espinosa et al., 2016) and may contribute to this disagreement in conclusions regarding treatments. Since a natural model was used here, it is likely that natural prion infections of ruminants involve mixtures of strains rather than a single strain (Mathiason, 2017), but it is necessary to emphasize that it would much better reflect reality.

In conclusion, we would like to reassert the essential requirement of using natural models to provide reliable results. It constitutes a powerful *in vivo* approach to assess the activation of glial cells by anti-inflammatory therapy in a trustworthy and quantitative manner. However, it is also much more time consuming and entails inherent difficulties and uncontrollable factors, mainly the exact time of natural infection in the field. Transgenic models have demonstrated poor representativeness of natural disease progress; some examples include studies that sought to delay the progress of amyotrophic lateral sclerosis (ALS) in a murine model (Kriz et al., 2002). Unfortunately, it worsened symptoms in clinic phase III in ALS-affected humans (Gordon et al., 2007) or epidemiological studies with drugs that managed to reduce the risk of developing PD and AD (Choi et al., 2009), while clinical assays in sick patients failed (Aisen et al., 2003).

Taking into consideration the overall results presented here and that some authors postulated that anti-inflammatory therapeutic approaches could be combined with other strategies to achieve improved therapeutic effects (Burwinkel et al., 2004), combining this strategy with a natural model, such as here, may confer an appropriate starting point to advance the subject.

**CONCLUSIONS**

The therapeutic approach tested in this work directly influences astroglial responses in healthy animals. However, no effect in the clinical stage of scrapie has been demonstrated here, likely due to an impaired astroglial response in affected animals. Our experimental results point to a neuroinflammation-augmenting effect of DEX in control animals. Therefore, new information has been obtained in this study. The present data show advances regarding the role of glial cells in scrapie. Moreover, although treatment did not seem to be clinically relevant to disease progress when clinical signs had already begun, the evident extension of survival in one case was hopeful.

We and others have shown the involvement of immune response in scrapie progression. This study also confirms the occurrence of neuroinflammation in neurodegeneration, as previously

described by Ransohoff, 2016. DEX induces rapid degranulation of mast cells (important cells of neuroinflammatory pathogenesis), which release pro-inflammatory molecules promoting activation of microglia and astrocytes. The increase of GFAP mRNA levels and decrease of AIF1 gene expression may show an effect of a chain reaction, where DEX induces an autocrine / paracrine cell signaling in which mast cells first followed by activation of microglia. Consequently, the activation of microglial cells was reduced, AIF1 gene expression was decreased and astrocyte activation was increased. Further studies focusing on these mast and glial cells would provide insight into the pathogenesis of scrapie in CNS and contribute to understanding the relationship between prions and the immune system.

In the future, how GC act on different cellular types and encephalic areas to produce such different immunomodulatory results in prion diseases should be determined. Collectively, these results reinforce the importance of performing basic research in order to better understand the unexpected capacity of GC to enhance aspects of CNS inflammation in neurodegenerative diseases.

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# ESTUDIO Nº 3:

## NEUROIMMUNE RESPONSE MEDIATED BY CYTOKINES IN NATURAL SCRAPIE AFTER CHRONIC DEXAMETHASONE TREATMENT

# ESTUDIO Nº 3: NEUROIMMUNE RESPONSE MEDIATED BY CYTOKINES IN NATURAL SCRAPIE AFTER CHRONIC DEXAMETHASONE TREATMENT

**ABSTRACT**

The actual role of prion protein - induced glial activation and subsequent cytokine secretion during prion diseases is still incompletely understood. The final aim of this study is to assess the effect of an anti-inflammatory treatment with dexamethasone on different cytokines released by neuroglial cells that are potentially related to neuroinflammation in natural scrapie. This study emphasizes the complex interaction existent among several pleiotropic neuromodulator peptides and provides a global approach to clarify neuroinflamamtory process in prion diseases. Additionally, an impairment of communication between microglial and astroglial populations mediated by cytokines, mainly IL-1, is suggested. The main novelty of this study consists of that it is the first one assessing *in situ* neuroinflammatory activity in relation with chronic anti-inflammatory therapy, gaining relevance because it is based on a natural model. The cytokine profile data suggest the activation of some neurotoxicity-associated route. Consequently, targeting such pathway might be a new approach to modify damaging effects of neuroinflammation.

**Key words:** scrapie, cytokine, dexamethasone, neuroinflammation, prion diseases.

**INTRODUCTION**

Scrapie is considered the prototype of prion diseases, that are a group of neurodegenerative diseases caused by the conversion of a cellular protein into a pathological isoform called prion.

Nowadays, neuroinflammation is a widely accepted concept in neurodegeneration, particularly in prion diseases (Burwinkel et al., 2004, Pasquali et al., 2006, Marcos Carcavilla et al., 2007, Servida et al., 2007). The neuroinflammatory process is defined as the prolonged activation of neuroglial cells with the corresponding production of inflammatory cytokines (Schwartz and Deczkowska, 2016). Consequently, there is a particular interest in investigating the role of innate and adaptive immune system in several neurodegenerative disorders, being neuroglia a key element in the neuropathological process (Verkhratski et al., 2012; Burda and Sofroniew, 2014; Pekny et al., 2016).

A relevant number of studies have proposed a crucial role for cytokines as neuroinflammatory mediators in the cellular communication in these prion diseases (Campbell et al., 1994; Williams et al., 1994, 1997; Peyrin et al., 1999; Veerhuis et al., 2002; Brown et al., 2003; Marcos Carcavilla et al., 2007). The detection of these cytokines was described coinciding with the onset of clinical signs, both in murine model (Campbell et al., 1994) and Creutzfeldt-Jacob disease (CJD) (Kordek et al., 1996). Moreover, a recent study has described the presence of several genes implicated in inflammation that are upregulated in early phases of prion infection (Carroll et al., 2015). Nevertheless, although an altered profile of inflammatory intermediaries has been evidenced in some experimental murine models (Campbell et al., 1994; Liberski et al., 1995; Kordek et al., 1996; Cunningham et al., 2002; Brown et al., 2003; Schultz et al., 2004; Thackray et al., 2004; Tribouillard Tanvier et al., 2009), scarce studies have focused on *in situ* tisular expression of these proteins (Williams et al., 1994; Cunningham et al., 2005) and none of them on a natural model.

Previous studies developed in scrapie affected animals have led to conclusions about the glial role in the neurodegenerative progress which resulted extrapolable not only to other prion but also neurodegenerative disorders (Hernández et al., 2014; Monzón et al., 2018; Garcés et al., 2019). More recently, this same *in vivo* model has been used in order to assess the changes of activation of glial cells associated with an anti-inflammatory therapy (Guijarro et al., 2020). This study constituted a powerful approach to the involvement of immune response in this neurodegenerative disease, confirming the occurrence of neuroinflammation in neurodegeneration. Specifically, a potential failure of astrocytes and a stimulation of phagocytosis of prion protein deposits by microglia were evidenced after dexamethasone

(DEX) treatment. To go in depth to interglial communication mediated by cytokines constitutes one main tool for advance in the knowledge about how these mediators are really involved in neuroinflammatory mechanisms contributing to neurodegeneration (Morales et al., 2014; DiSabato et al., 2016; Ransohoff, 2016; Kempuraj et al., 2016). It is indispensable to study the possible alteration of glial crosstalk that might enhance instead of prevent neuronal damage. Thus, it could be a crucial target for therapeutic approaches in prion diseases (Garwood et al., 2011). On the basis of all of this, to investigate the possible alterations of *in situ* cytokine expression in brain samples from animals naturally affected by scrapie and DEX treated is proposed here as first step towards this goal.

All in all, the actual role of prion protein - induced glial activation and subsequent cytokine expression during prion diseases is still incompletely understood. Provided that there are clear evidences to think that cytokines could be significant factors in the progress of neurodegeneration in this group of diseases, the specific aim of this study is to assess the effect of an anti-inflammatory treatment on different cytokines potentially related to inflammation. Both immunohistochemical and expression pattern of different pro-inflammatory and anti-inflammatory ones in several brain regions from treated and non-treated scrapie affected sheep are analysed in this study. To determine whether these proteins represent a relevant target in immunopathogenesis of neurodegeneration is finally intended.

**MATERIAL AND METHODS**

All the following experimental procedures were previously approved by the Ethical Committee of University of Zaragoza (Reference number: PI41/16). All efforts were made to minimize animal suffering during the experiments and to reduce the number of animals used.

The experimental analyses were performed on samples coming from animals where glial activation associated with DEX treatment had been assessed (Guijarro et al., 2020). A total of 25 sheep were included in this study: 10 healthy control (of which 4 treated and 6 non-treated) and 15 clinical scrapie Rasa Aragonesa ewes (10 treated plus 5 non-treated). Healthy controls were considered essential in order to specifically observe the effect of treatment in normal conditions in ovine species. After euthanasia with intravenous pentobarbital injection, necropsy of each sheep was performed and 80 samples were subsequently collected and distributed for different studies. One hemisection from each sample was fixed by immersion in

4% paraformaldehyde for immunohistochemical studies and the other hemisection was frozen at -80ºC for molecular studies (RT-qPCR).

*IMMUNOHISTOCHEMICAL TECHNIQUES*

Immunohistochemistry (IHC) was carried out in order to assess *in situ* neuroinflammatory profile associated with DEX treatment in all sheep. It was compared with non-treated sheep group in four encephalic areas (frontal cortex: Fc, cerebellum: Cb, obex: O and medulla oblongata: MO).

Prior 4 µm sectioning, paraffin-embedding of fixed samples was developed. After specific pre-treatments for antigen retrieval, specific immunohistochemical protocols by using specific primary antibodies against those cytokines or their receptors mainly studied in literature related (to our knowledge, IL-1α, IL-1R, IL-2R, IL-6, IL-10R, TNFαR and IFNγR) were applied. The EnVision system (DAKO, Glostrup, Denmark) and diaminobenzidine (DAB; DAKO, Glostrup, Denmark) were used as the visualization system and chromogen respectively. Hematoxylin counterstaining and mounting in DPX was finally performed on all sections.

All slides were analysed by two independent observers scoring the intensity of immunostaining from 0 (absence) to 4 (maximum presence) by counting positive cells (Romero-Trevejo et al., 2010) in 5 microscopic fields in each brain region examined. Moreover, provided that cerebellum has been proposed as a pseudo reference region to detect neuroinflammation (Lyoo et al., 2015), close attention was paid to different profiles of cytokine distribution in this brain region. The focus was on Purkinje cells based on previous results referring this neuronal type as the most damaged while they are the most protected neurons in this area (Hernández et al., 2014; Sarasa et al., 2015; Guijarro et al., 2020).

Table 1 summarises all the primary antibodies and the protocols applied as following described.

**Table 1.** Antibodies used for immunohistochemical techniques and retrieval method applied for each one.

| Antibody | Antigen | Type | Dilution | Retrieval method | Source |
|---|---|---|---|---|---|
| **IL-1 alpha** | IL-1 | Polyclonal | 1:100 | Autoclave 121ºC (citrate buffer 10%) | ThermoFisher |
| **Anti-IL-1RN** | IL-1R | Polyclonal | 1:100 | Autoclave 121ºC (citrate buffer 10%) | Sigma |
| **IL-2R.1** | IL-2R | Monoclonal | 1:1.000 | PTLink 96ºC | ThermoFisher |
| **8H12** | IL-6 | Monoclonal | 1:40 | Autoclave 121ºC (citrate buffer 10%) | ThermoFisher |
| **OTI1D10** | IL-10R | Monoclonal | 1:250 | PTLink 96ºC | ThermoFisher |
| **Ber-H2** | TNFαR | Monoclonal | Ready to use | PTLink 96ºC | Dako |
| **IFNGR1** | IFNγR | Polyclonal | 1:200 | Autoclave 121ºC (citrate buffer 10%) | ThermoFisher |

- IL-1, IL-1R, IL-6 and IFNγR detection

A pre-treatment consisting of hydrated heating at 121ºC in citrate buffer 10% for 20 min preceded the endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min and incubation overnight 4ºC with different primary antibodies: polyclonal IL-1α (1:100; ThermoFisher Scientific, Waltham, MA, USA), polyclonal IL-1RN (1:100, Sigma, USA), monoclonal 8H12 (1:40; ThermoFisher Scientific, Waltham, MA, USA) or polyclonal IFNGR1 (1:200, ThermoFisher Scientific, Waltham, MA, USA).

- IL-2R, IL-10R and TNFαR detection

A pre-treatment consisting of hydrated heating at 96ºC in citrate buffer 10% for 20 min preceded the endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min and incubation with different primary monoclonal antibodies: IL-2R.1 (1:1.000, overnight 4ºC; ThermoFisher Scientific, Waltham, MA, USA), OTI1D10 (1:250, overnight 4ºC; ThermoFisher Scientific, Waltham, MA, USA) or Ber-H2 (ready to use, 30 min RT; DAKO, Glostrup, Denmark).

*RT-qPCR*

Cerebellum and frontal cortex frozen tissues from treated and non-treated scrapie animals were included in the following comparative molecular analysis for some inflammatory markers.

- RNA purification

The purification of RNA was performed following the instructions of the supplier (RNeasy Lipid Tissue Mini kit, Qiagen, GmbH, Hilden, Germany). RNA integrity and 28S/18S ratios were determined with the Agilent Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA). Samples were treated with DNase digestion, and RNA concentration was evaluated using a NanoDrop Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

RNA samples with OD 260/280 ratios close to 5.0 were selected for reverse transcription. Finally, a total of 5 treated and 4 non-treated clinical sheep were included in this molecular analysis.

- Retrotranscription

Retrotranscription of RNA into cDNA was performed according to the manufacturer's instructions (High-Capacity CDNA Reverse Transcription Kit, Applied Bio systems).

- RT-qPCR

Gene expression of IL-1, IL-6, IL-10Ra, IL-10Rb and IFNγ in both clinical non-treated and treated sheep was assessed. The parameters of the reactions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Data were assessed using the ΔΔCt method, using Hypoxanthine Phosphoribosyl transferase 1 (HRPT-1) and β-glucuronidase (GUS-β) as reference genes.

Table 2 shows TaqMan probes used for these molecular studies.

**Table 2.** Taqman probes used for RT-qPCR analysis.

| Gene | Full name | Reference | Source |
|---|---|---|---|
| Gus-β | β-glucuronidase (reference gene) | Oa04828868_m1 | ThermoFisher |
| HPRT-1 | Hypoxanthine Phosphoribosyltransferase 1 (reference gene) | Oa04825272_gH | ThermoFisher |
| IL-1α | Interleukin 1 alpha | Oa04658681_m1 | ThermoFisher |
| IL-6 | Interleukin 6 | Oa04656315_m1 | ThermoFisher |
| IL-10Ra | Interleukin 10 receptor alpha | Oa04822455_m1 | ThermoFisher |
| IL-10Rb | Interleukin 10 receptor beta | Oa04894070_m1 | ThermoFisher |
| IFNγ | Interferon gamma | Oa04657364_m1 | ThermoFisher |

*STATISTICAL ANALYSIS*

For IHQ results, the normality of distribution was first assessed by Kolmogorov-Smirnov test. The non-parametric Mann-Whitney *U* test was used to assess quantitative differences between non-treated and DEX treated groups.

Data of RT-qPCR were evaluated by Student's *t* test after assessing normality also by Kolmogorov-Smirnov test.

SPSS software (SPSS Statistics for Windows, Version 17.0) was used for these analyses and significance in all cases was taken at $*p < 0.05$. All graphs were performed with GraphPad Prism 6.0. Data presented in figures are expressed as means and the standard error of the mean (mean +/- SEM).

**RESULTS**

*IMMUNOHISTOCHEMISTRY*

- IL-1

DEX treated controls always displayed higher intensity for IL-1 staining compared to the untreated ones. However, clinical treated animals showed lower differences with their respective untreated group; they even reversed (Figure 1A).

Mann Withney *U* test revealed significant effects of DEX in treated controls compared to untreated ones, showing an increase of IL-1 immunostaining in MO (**p* = 0.024). Meanwhile, no changes were detected for clinical scrapie sheep depending on the treatment (Figure 1B).

In Cb, immunostaining for IL-1 was widespread in all layers (Figure 2A).

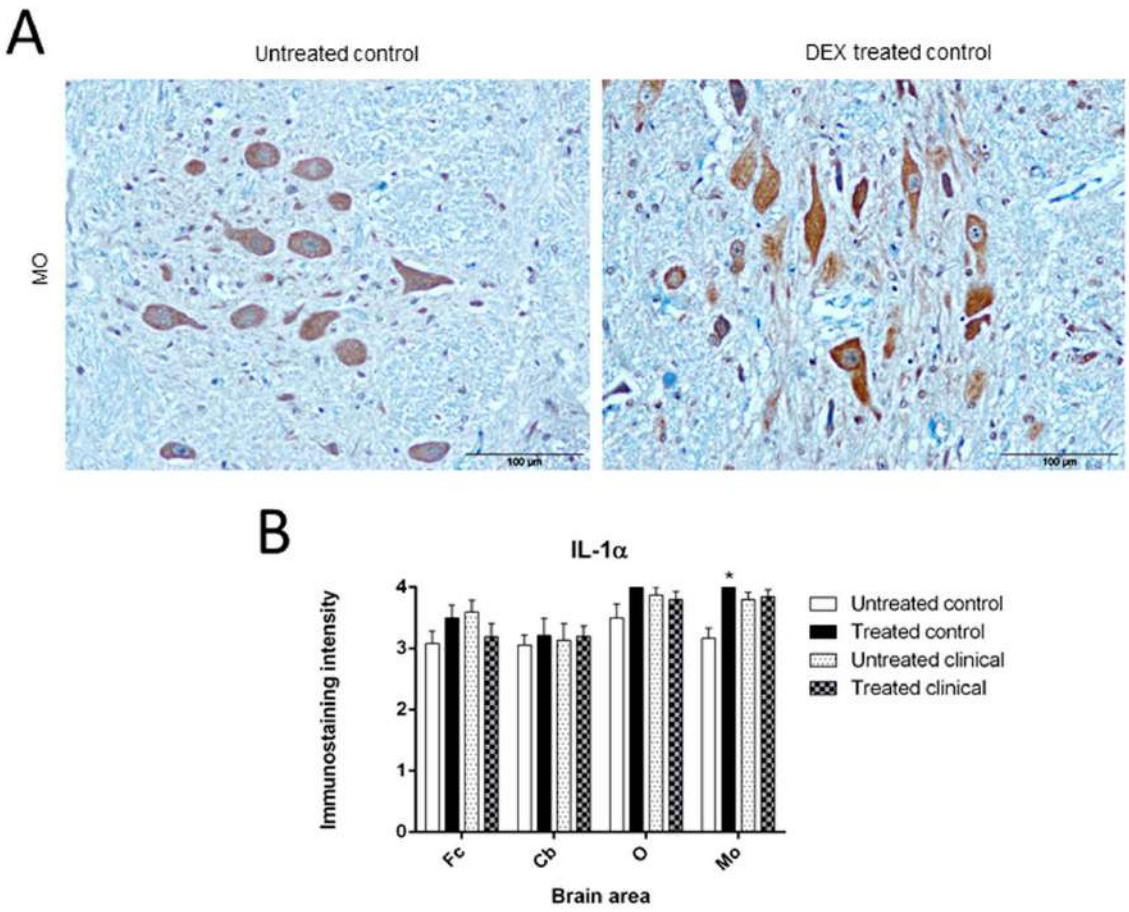


**Figure 1.** IL-1 immunostaining. **(A)** Note the evident higher intensity of immunostaining in MO from DEX treated control sheep compared to an untreated one. Scale bars: 100 µm. **(B)** A significant effect of DEX was observed in MO from treated controls (**p* < 0.05). However, at clinical stage, no significant changes were detected despite of the treatment.

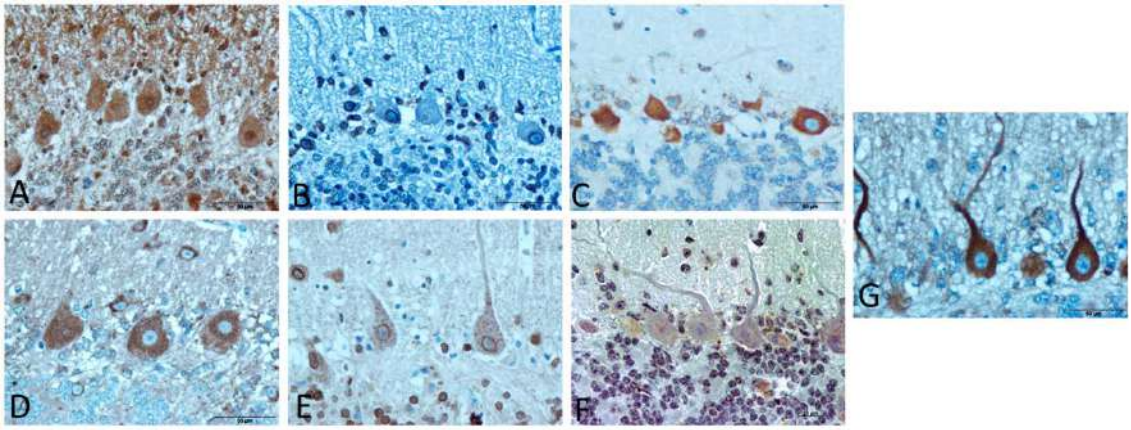


**Figure 2.** Morphological findings in cerebellum immunostained with different primary antibodies. **(A)** Immunostaining for IL-1 was widespread in all layers. **(B)** IL-1R immunoreactivity was located mainly surrounding Purkinje cells, suggesting a morphology consistent with astrocytes. **(C)** IL-2R was mainly found in cytoplasm of Purkinje cells and cells appearing astrocytes. **(D)** IL-6 immunostaining was mainly present in Purkinje cells as intracytoplasmatic staining, as well as stained cells resembling glial cells in both granular and molecular layers. **(E)** IL-10R immunostaining appeared spot intracytoplasmatic in Purkinje cells as well as granular and other cells with astrocytic morphology. **(F)** Purkinje cells expressed TNFαR in a very lower extent than the rest of markers. **(G)** IFNγR immunostaining pattern was mainly localized in the cytoplasm and dendritic spines of Purkinje cells. Scale bars: 50 µm.

- IL-1R

In general, immunostaining was lower in treated animals compared to the non-treated ones, except for controls in Fc, where it was exactly the opposite (higher). These differences evidently increased in O and Cb at clinical stage (Figure 3A).

Despite no statistically significant changes were observed for this marker, a trend to a reduction in immunostaining in Cb from animals at clinical stage was detected (#$p$ = 0.082) (Figure 3B).

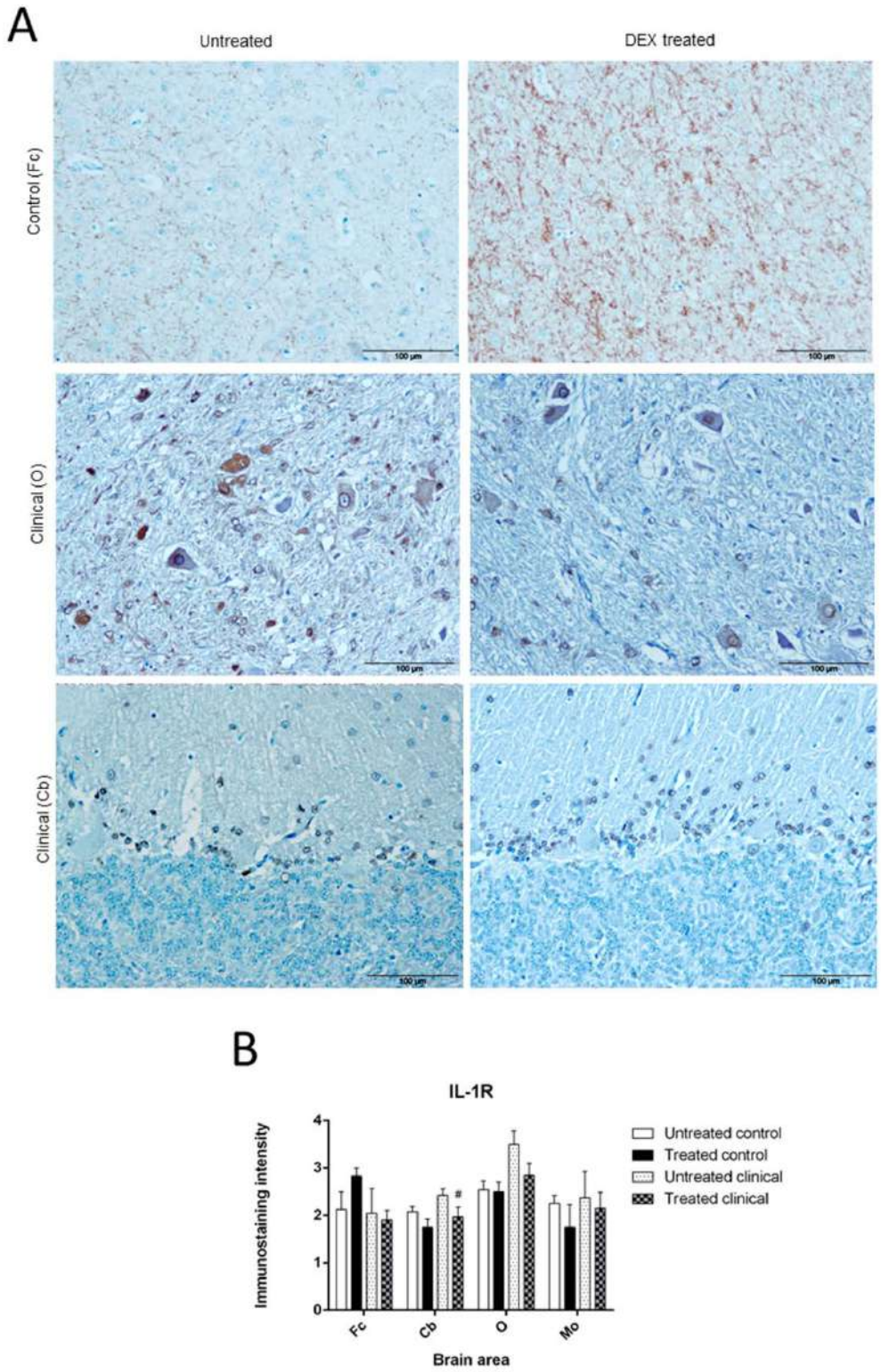


**Figure 3.** IL-1R immunostaining. **(A)** Differences observed after treatment in Fc of control sheep and O and Cb at clinical stage are illustrated. Scale bars: 100 µm. **(B)** While a subtle reduction of IL-1R in treated control group was observed in the rest of areas examined, in Fc it increased after DEX treatment. At clinical stage, a decrease of immunostaining in O and a trend (#$p$ = 0.082) to reduction in Cb were detected after treatment.

IL-1R immunoreactivity in Cb was nearly exclusively located in cells surrounding Purkinje cells, suggesting morphology consistent with specific astrocytes (Figure 2B).

- IL-2R

It is only relevant to point out that Fc showed an exacerbated increase of reactivity against this cytokine regardless treatment and disease in comparison with the other brain areas.

No statistically significant differences were found regarding treatment in both control and clinical groups (Figure 4A).

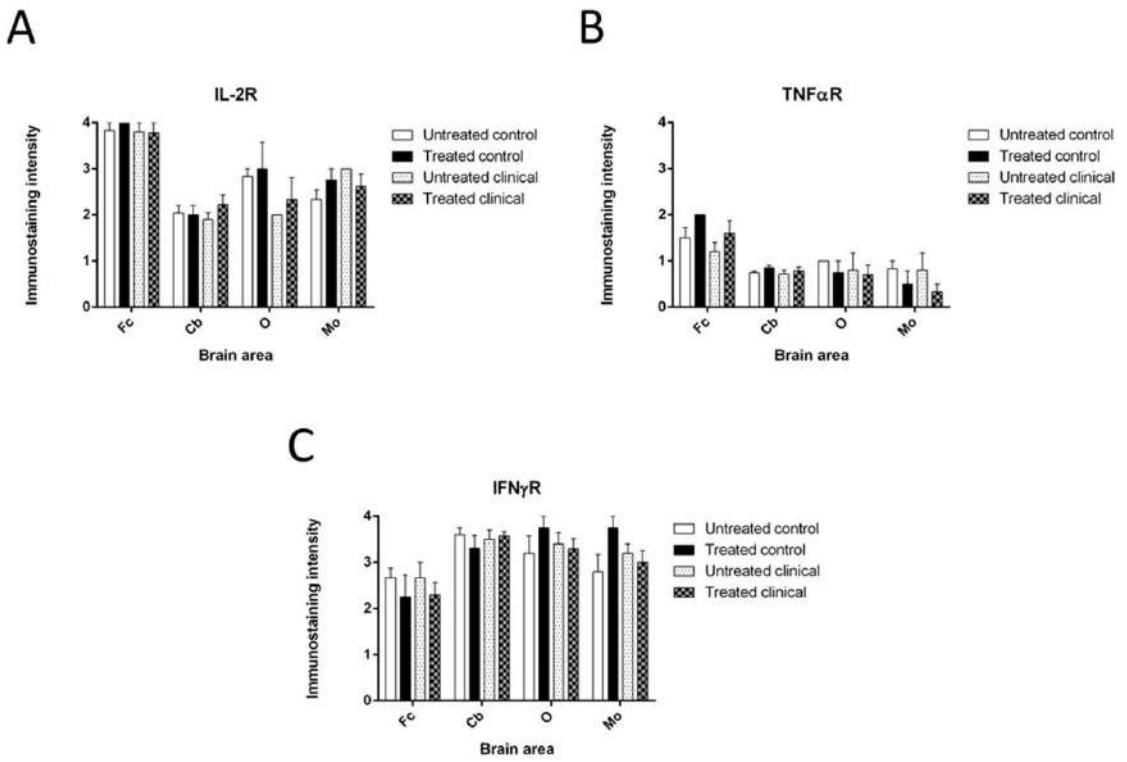


**Figure 4.** No significant differences were found either in control or in clinical groups for immunostaining intensity for **(A)** IL-2R **(B)** TNFαR or **(C)** IFNγR. A higher intensity in O and MO after treatment was evidenced in controls that reversed in DEX clinical animals.

In Cb, IL-2R was limited to cytoplasm of Purkinje cells as well as cells with an astrocytic appearance (Figure 2C).

- IL-6

Immunostaining for IL-6 in DEX treated controls was higher in all brain areas compared to untreated controls, except in O, where the labelling intensity was lower. This reduction in O was more evident at clinical stage of disease (Figure 5A).

The increase of immunostaining intensity reached significance in Fc of control group when animals were DEX treated (**p* = 0.040). Meanwhile, the immunoreactivity for this cytokine showed a trend to decrease in control animals (#*p* = 0.071) that converted into significant in O from clinical group (**p* = 0.041) (Figure 5B).

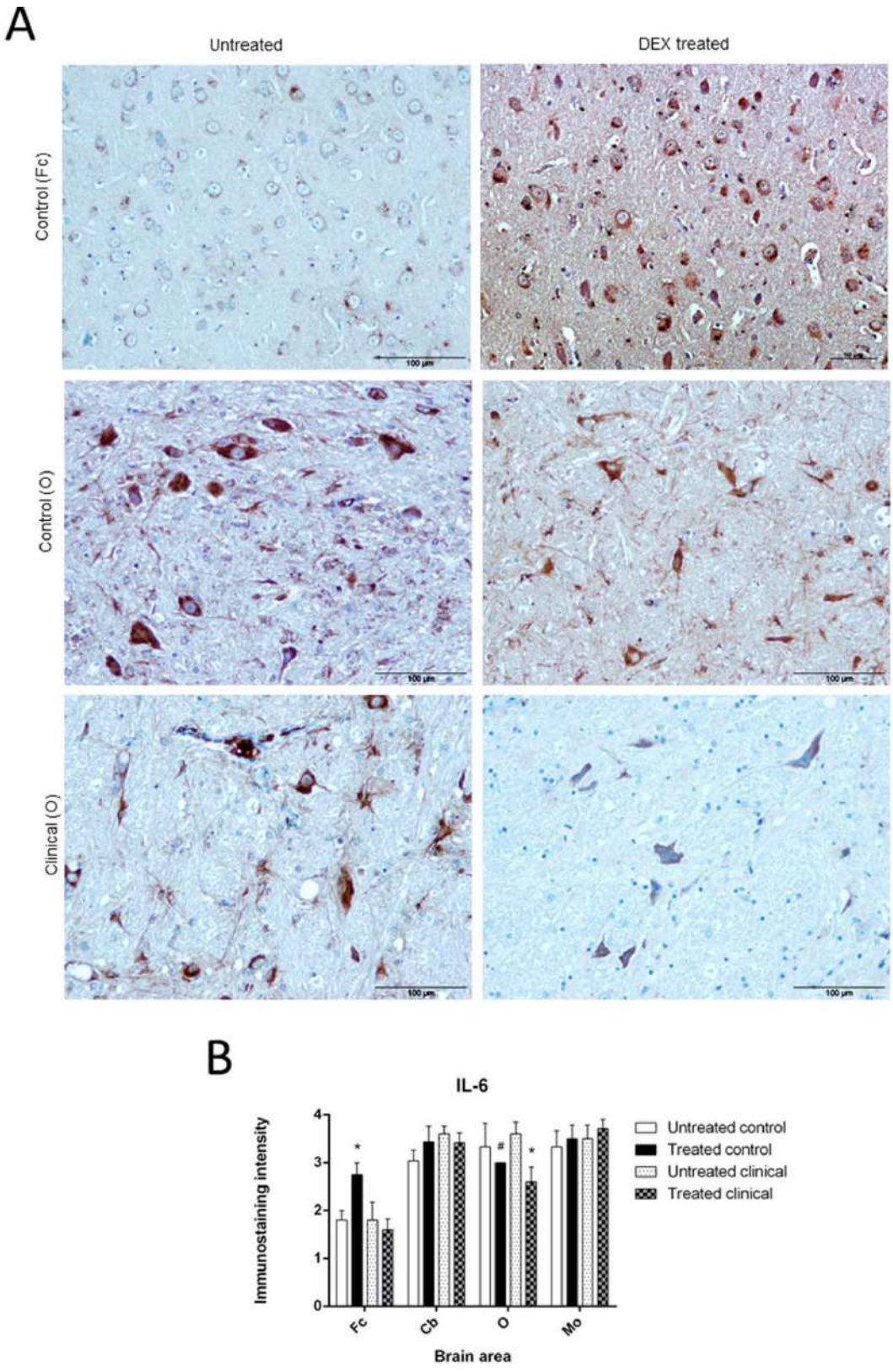


**Figure 5.** IL-6 immunostaining. **(A)** Micrographs represent an increase of IL-6 immunoreactivity in Fc in treated controls while slightly reduced in both treated groups in O. Scale bars: 100 µm. **(B)** DEX control group presented a significant increase in Fc (**p* < 0.05) and a trend to a decrease in O (#*p* = 0.071). It was significantly decreased in this area when clinical group was treated (**p* < 0.05).

This neuromodulator peptide was mainly present in Purkinje cells as intracytoplasmatic staining (Figure 2D). Moreover, the cytokine detected was present in specific cells resembling glial cells in both granular and molecular layers.

- IL-10R

Immunostaining for this marker was the highest in all assessed samples in both control and clinical groups, regardless treatment; but especially in Fc, where the highest score was reached regardless treatment and disease.

In the same line, although not significant, a trend to a decrease (#$p$ = 0.074) in Cb of clinical sheep after treatment was observed (Figure 6A). In general, statistically, immunostaining for IL-10R did not reveal any changes between groups after treatment ($p$ > 0.05). Nevertheless, a very relevant decrease in intensity in O of treated clinical animals was evidenced to reach lower intensity than untreated while it was higher in controls (Figure 6B).

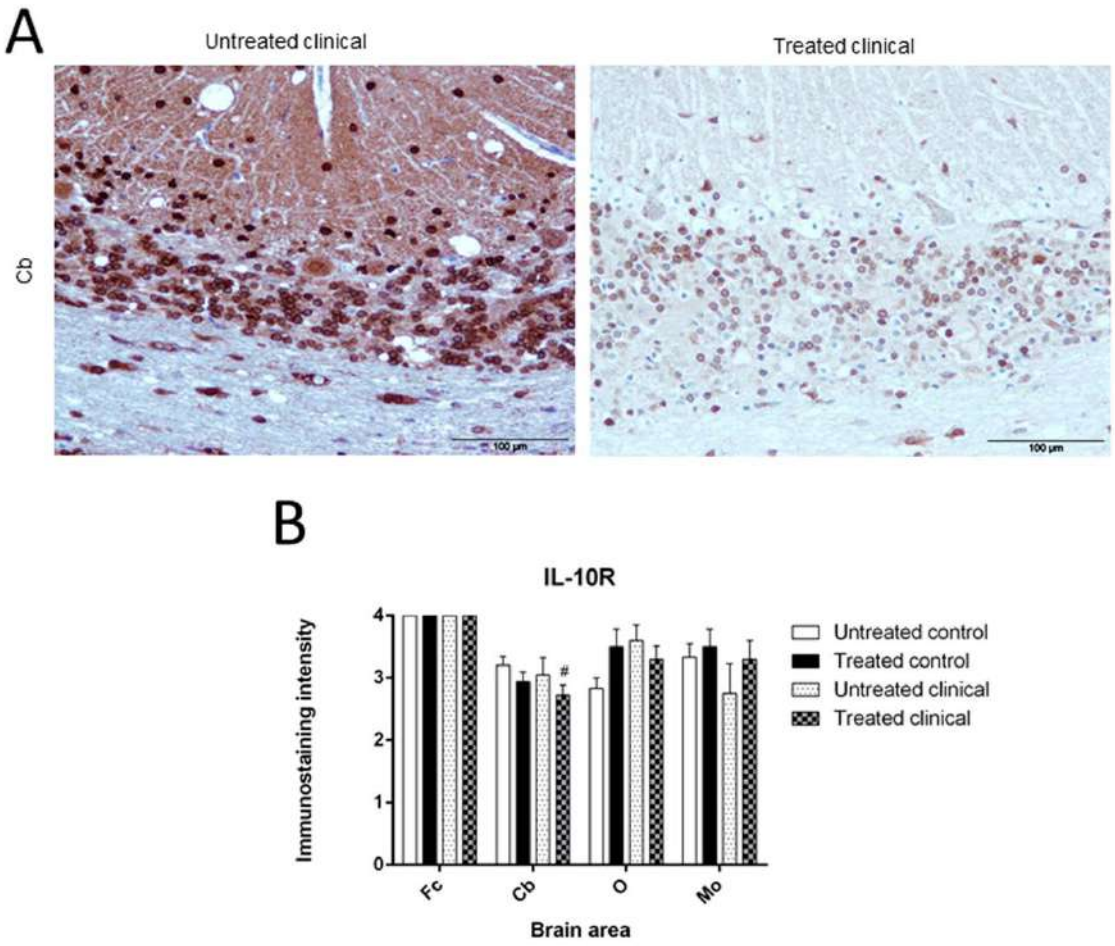


**Figure 6.** IL-10R immunostaining. **(A)** Decrease in intensity in Cb of treated clinical sheep is illustrated. Scale bars: 100 µm. **(B)** No significant changes were detected after treatment in control group, only a higher increase in O was outstanding. No significant changes were either observed in clinical stage after treatment, just a trend to lower intensity in Cb of treated clinical sheep ($p$ = 0.074).

This marker stained Purkinje neurons consistent of spot intracytoplasmic pattern. Additionally, it showed a high intensity in granular cells and some others with astrocytic morphology (Figure 2E).

- TNFαR

In general, immunostaining for TNFαR was less intense in all brain areas compared to the rest of cytokines assessed here.

The pattern of TNFαR immunoreactivity was similar in control and clinical animals, being no significant after treatment in any case ($p$ > 0.05) (Figure 4B).

Morphologically, Purkinje cells in Cb expressed this receptor in a very lower extent than the rest of cytokines assessed here (Figure 2F).

- IFNγR

Whereas in DEX treated controls the immunostaining was higher in O and MO, this difference just reversed in DEX treated clinical. On the contrary, immunostaining intensity increased in Cb from treated controls to exceed untreated animals when they were affected by clinical disease.

Nevertheless, no significant differences in intensity levels were found between treated and non-treated groups in any encephalic area for this marker ($p > 0.05$) (Figure 4C)

In Cb, its immunostaining pattern was mainly located in the cytoplasm and dendritic spines of Purkinje cells (Figure 2G).

*RT-qPCR*

Significantly, DEX treatment resulted in more than a three-fold increase in IFNγ mRNA levels in Fc of clinical treated sheep (*$p$ =0.036) (Figure 7A).

Regarding Cb, no statistically significant changes were found in the mRNA expression of any cytokine analysed here, although all of them were higher at clinical treated (Figure 7B).

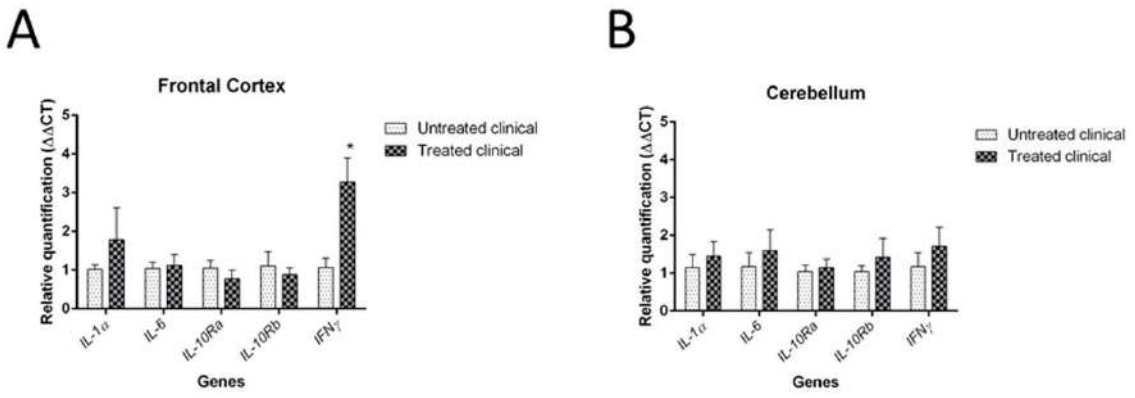


**Figure 7.** Results of gene expression by RT-qPCR in clinical sheep. **(A)** In Fc, an increase of IFNγ mRNA levels was observed (*$p$ = 0.036). **(B)** In Cb, no significant changes were found in the mRNA expression of any cytokine analysed in this study, but all of them were slightly higher in clinical stage after DEX treatment.

**DISCUSSION**

A great number of suggestions have lead to think about the existence of a mixed inflammatory profile in prion diseases contributing to immune response (Vincenti et al., 2015; Perry, 2016; Obst et al., 2017); on one hand, some studies described a limited pro-inflammatory response, with no increase of IL-1 and TNFα (Walsh et al., 2001; Cunningham et al., 2002, 2005; Perry et al., 2002); on the other hand, some others described an increase of some factors, these same cytokines among them (Campbell et al., 1994; Williams et al., 1994; Kordek et al., 1996). Actually, it has been described the presence of an impaired response of cytokines in mice orally infected with scrapie, imitating the natural route of entry (Romero-Trevejo et al., 2010). However, these previous investigations in murine models have not been able to reach a consensus regarding inflammatory profile in prion diseases because level expressions vary depending on the strain of mice and different combinations of scrapie inoculum (Walsh et al., 2001; Brown et al., 2003) as well as different disease stages and detection techniques (Obst et al., 2017). Moreover, direct detection of cytokines in brain by IHC has been achieved only by some authors probably because of the challenge associated with their detection as labile soluble proteins present at low concentrations (Boche et al., 2013). A novel *in situ* cytokine profile is described here, slightly different to what has been previously done, maybe due to that the natural model was used here. It is crucial to highlight the relevance of results from natural models, which are more reliable than those from experimental models, providing a fairer reflexion of reality.

The findings presented in our previous study supported an impaired astroglial response at clinical stage of scrapie, suggesting astroglial paralysis (Guijarro et al., 2020), as it was recently described in late stages of Alzheimer's disease (AD) (Verkhatsky et al., 2019). Our next goal consisted of providing specific information about the mechanisms underlying cytokine release from glial cells. For that purpose, the effect of long-term DEX treatment in sheep with natural scrapie as well as in healthy controls was examined here in order to determine whether this treatment would change the levels of neuroinflammatory markers in this neurodegenerative disease. To clarify the relationship between neurodegeneration and neuroinflammation is finally intended.

As it has been reported that neuroinflammation in prion diseases is region-dependent (Llorens et al., 2014), this study was designed assessing four different brain areas. Moreover, no studies about *in situ* variations in cytokine levels after glucocorticoid (GC) treatment in prion diseases have been previously published, despite it is well-known that chronic administration of GC inhibits both innate and adaptive immune systems, reducing a great variety of pro-

inflammatory cytokines (Forster et al., 2006), such as IL-1, TNFα (De Bosscher et al., 2003), IL-6 and IFNγ (Munck et al., 1984; Guerne et al., 1990). In most cases, chronic exposition to GC suppress mediators; however, on some occasions GC can potentiate such immunity (Bowers et al., 2008), especially in central nervous system, which can exacerbate neuronal death (Sorrells et al., 2009). Thus, in some cases of murine AD, neurodegeneration and exacerbation of pro-inflammatory mediators were observed after chronic exposition to this anti-inflammatory drug (Hu et al., 2016). Consequently, the doses and duration of treatment is of huge importance to have one or another response of immune system. Additionally, acquired resistance with absence of response to treatment has also been described in rheumatoid arthritis (Kirwan, 2007).

Here we show that the low dose of DEX chronically administered to healthy animals significantly increased the secretion of IL-1, specifically in one of the brain areas examined (MO). The early over-expression of IL-1 has been suggested as a candidate to contribute to the development of murine (Brown et al., 2003; Burwinkel et al., 2004; Schultz et al., 2004) and ovine scrapie (Marcos-Carcavilla et al., 2007) as well as AD (Mrak et al., 1995; Akiyama et al., 2000; Griffin and Mrak, 2002; Sastre et al., 2006). This increase has been also described in clinical and terminal stage in another scrapie murine model (Cunningham et al., 2005). Furthermore, it is a well-known fact that expression of IL-1 induces oxide nitric synthase (iNOS) (Serou et al., 1999) and this production is involved in damage and neuronal death (Zheng et al., 2001). Enhanced neurodegeneration was observed when anti-inflammatory treatment was provided before the neurodegenerative lesion itself in rats (Dinkel et al., 2003). Taking into account this enhancement, DEX could be speculated to present a possible neurotoxic effect in control sheep. As our previous study about glial activation response after DEX treatment showed an increment in glial fibrillary acidic protein (GFAP) levels in treated controls, mainly observed also in MO (Guijarro et al., 2020), higher IL -1 levels could be related with this astrocytic activation, provided that some authors have closely related IL-1 to astrogliosis (Brown et al., 2003; Burwinkel et al., 2004). By opposite, no variations in the level of this mediator were evidenced after treatment when animals were scrapie affected. This could reinforce the hypothesis that an astroglial dysfunction exists in scrapie progress (Guijarro et al., 2020). Maybe a possible impairment of communication would be suggested between microglia and astroglia in clinical scrapie because IL-1 is not increased in this group of animals although microglial population is shown to be activated (Guijarro et al., 2020).

In this natural model, the expression of receptor for this IL-1 was slightly reduced in all treated cases (both control and clinical) except in Fc of controls. As that deficiency of IL-1R was

demonstrated to prolongue incubation time (Tamgüney et al., 2008), delay disease onset and expand survival period of affected mice (Schultz et al., 2004), this could point to the possibility of a neuroprotective role for DEX in both control and clinical sheep. Nevertheless, there are other studies in which IL-1R *knockout* mice inoculated with prions did not have any effect on disease (Carroll et al., 2015). The presence of neurotoxic reactive astrocytes induced by IL-1 and TNFα released by microglia has been described (Liddelow et al., 2017). Furthermore, a recent study shows that microglia has the power to autorenew itself and IL-1R signalling is implicated in this proliferative process (Bruttger et al., 2015). It could be therefore speculated that DEX prevents the self renewal of microglia, fact that might be again related to the impairment of communication with astroglia. On the other hand, IL-1R immunoreactivity was located mainly surrounding Purkinje cells in cerebellum and appeared morphology consistent with that of astrocytes, in agreement with previous studies in murine models (Williams et al., 1994, 1997; Kordek et al., 1996; Brown et al., 2003). Indeed, IL-1R has been described to be expressed by microglia, astrocytes and neurons in rodents (Ban et al., 1991; French et al., 1999), but particularly by cerebellar Purkinje neurons (French et al., 1999). Importantly, it appears that IL-1R in neurons mediates the fast changes in neuronal excitability induced by IL-1, while IL-1R in astrocytes mediates effects of IL-1 on neuronal survival (Ravizza and Vezzani, 2006). Therefore, evidence of the complexity of the IL-1 ligand / receptor, whose balance depends on the phase of the inflammatory response, is shown here (Spulber et al., 2009). Given that only few activated IL-1R per cell are sufficient for activation of the target cell (Dinarello, 1996), this is of particular relevance for their signalling system. All in all, it is strongly evidenced here that both IL-1 and IL-1R play a significant role in progress of natural ovine scrapie.

In regards to IL-2, it has been shown that this mediator can ameliorate amyloid pathology in mice with AD (Alves et al., 2017). Nevertheless, nor this mediator nor its receptor have been analysed in prion diseases field before. Some trials revealed IL-2 anti-inflammatory effects (Saadoun et al., 2011). However, in the present study, no differences were found regarding treatment in both control and clinical groups, thus not attributing a relevant anti-inflammatory role of DEX regarding this cytokine. Fc has been shown to be the brain region more reactive against this mediator and similar to our study, it has been shown that immunorreactivity of IL-2 in brain of both controls and AD patients were widespread (Luber-Narod and Rogers, 1988). Morphologically, in Cb, its receptor was basically found in cytoplasm of Purkinje cells and astrocytes. Given that other authors have described that hippocampal neurons are rich in this receptor, which has been related to neuronal development (Sarder et al., 1993; Dansokho et

al., 2016), we could also suggest that IL-2 is involved in neuronal development of Purkinje cells in this encephalic area. Taken together, although DEX treatment has had no apparent effect in modulating the levels of this peptide, a role of IL-2 in prion diseases should not be discarded before developing further studies.

Whereas DEX caused a significant increase in IL-6 staining intensity in Fc of control group, which is in total agreement with the strong astrogliosis observed in treated controls in our previous study (Guijarro et al., 2020), the immunoreactivity for this cytokine was reduced in O of both treated control and clinical group. Of particular interest is the fact that over-expression of IL-6 has been shown to produce neurologic disease in mouse, activating astrocytes and microglia (Campbell et al., 1993). Moreover, Burwinkel et al., 2004 suggested that IL-6 might be implicated in terminal stage of scrapie since it activates astrocytes *in vivo* (Hafiz and Brown, 2000). Consequently, blocking of IL-6 has been proposed as a possible treatment against chronic inflammatory diseases (Gabay, 2006). Taking all this into account and given the evidence presented herein, DEX might act as neuroprotective for both control and clinical animals in one brain area (O) while it could be neurotoxic for controls in another one (Fc). Needless to say that IL-6 is a very peculiar cytokine that, depending on the context, exhibits two different behaviours: in acute inflammation models, is anti-inflammatory (Xing et al., 1998 Yasukawa et al., 2003), while it is pro-inflammatory in chronic inflammation models (Tilg et al., 1997; Yamamoto et al., 2000). In summary, IL-6 can act as both pro-inflammatory and anti-inflammatory having both neurotrophic and neurotoxic effects on neurons (Allan and Rothwell, 2001). Thus, although one study postulated that mice deficient in IL-6 gene did not have any effect in prion pathogenesis (Mabbot et al., 2000), we strongly support the idea of this neuromodulator peptide is relevant to prion diseases pathogenesis (Campbell et al., 1994; Williams et al., 1994, 1997; Kordek et al., 1996; Peyrin et al., 1999; Veerhuis et al., 2002; Brown et al., 2003; Marcos Carcavilla et al., 2007) as it has been described for prion-*like* diseases (Bauer et al., 1991; Strauss et al., 1992; Wood et al., 1993).

Immunostaining of the marker specific for IL-10R was the highest compared to the rest of cytokines, which is in accordance with the treatment applied, provided that this mediator has been described as a potent anti-inflammatory cytokine (Couper et al., 2008) that interferes with nuclear factor kB (NF-kB) activation and blocks the synthesis of other pro-inflammatory cytokines (Wang et al., 1995), thus receiving a considerable attention in neuroinflammation research (Lobo-Silva et al., 2016). Actually, Thackray et al., 2004 demonstrated an accelerated pathogenesis of prion diseases in absence of IL-10, with extension of survival time in mice (Tamguney et al., 2008), which attributes a protecting role of IL-10 in prion diseases.

Nevertheless, due to that no changes between groups after treatment have been detected (nor by IHC nor RT-qPCR), and even a subtle decrease in intensity of this mediator in some brain areas at clinical stage after treatment was observed in the present study, it cannot be completely confirmed the role as immunomodulator and anti-inflammatory of this cytokine in natural ovine scrapie, as it has been recently described in models of AD (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015).

Any changes were neither detected for TNFαR in any group regardless treatment. This supports studies that did not attribute a relevant role to this cytokine in scrapie progress (Mabbott et al., 2000) and prion pathogenesis (Prinz et al., 2002). On the other hand, TNFα has been described to be implicated in panencephalic CJD pathogenesis (Liberski et al., 1995), and an increment in incubation period in TNFα *knockout* animals has been also described (Mabbott et al., 2002). Indeed, it is a very peculiar mediator, provided that elevated (Zhao et al., 2003) and reduced levels (Cacabelos et al., 1994) of this peptide and its receptor have been described in AD. Consequently, either neuroprotective (Sakudo et al., 2003) or neurotoxic roles (Goetz et al., 2004) have been suggested by other authors (Scherbel et al., 1999). The discrepancies among studies regarding the role of this cytokine might be due to different experimental models. Thus, it is worth mentioning that, to our knowledge, this is the first study based on a natural model assessing *in situ* neuroinflammatory activity in prion diseases in relation with chronic anti-inflammatory therapy. As mentioned, the presence of neurotoxic reactive astrocytes induced by IL-1 and TNFα released by microglia has been observed (Liddelow et al., 2017). This fact encourages proposing again an impairment of communication between microglia and astroglia.

Regarding IFNγR intensity levels, a similar behaviour was demonstrated in all groups. However, immunostaining was slightly higher in some encephalic areas (O and MO) from controls after treatment, while this difference diminished and even reversed in treated clinical stage. Taking into account that microglia stimulates their division and adult neurogenesis by releasing IFNγ among other mediators (Kim and de Vellis, 2005; Sato, 2015), this finding could be reflecting an attempt to microglial division in some brain regions in controls. Meanwhile, no effect is proved at clinical stage of the disease by IHC. Regarding molecular expression, strikingly, we found a significant increase in the quantity of IFNγ mRNA by RT-qPCR in a specific area (Fc) of clinical treated sheep and these results could be reflecting the microglial expansion observed in previous studies in natural scrapie (Hernández et al., 2014; Guijarro et al., 2020). However, no statistically significant changes were found in the mRNA expression of any cytokine in Cb,

which agrees with the idea that neuroinflammation in prion diseases is region-dependent (Llorens et al., 2014). The discrepancies in results provided by molecular and IHC techniques are most likely due to the reduced number of samples available for RT-qPCR (9 samples in total, while 15 samples were used for IHC). In addition, as prolonged expression of IFNγ in central nervous system has been reported to lead to neuronal and glial cell damage (Corbin et al., 1996) and activated astrocytes have been described to have high levels of IFNγR in Parkinson's disease models, suggesting that this cytokine can mediate toxic effect of neighbouring neurons (Hashioka et al., 2009), DEX treatment could have had neurotoxic effect both in control and clinical animals. Further studies are necessary in order to clarify this discernible contradiction.

Concerning with observations focused on cell damage in Purkinje cells as the most damaged while the most protected neurons in cerebellum (Sarna and Hawkes, 2003; Hernández et al., 2014; Monzón et al., 2018; Guijarro et al., 2020), different immunostaining patterns of cytokines or receptors have been demonstrated in this study. Certainly, other authors found that immunostaining for cytokine receptors were higher in Purkinje cells than in other cells in mouse cerebellum (Nelson et al., 1999). Such distribution in mice is similar to that described in our study in sheep, confirming that cytokine receptors are present in high extent in Purkinje neurons in ovine cerebellum. This distribution could mean that the respective ligand of each receptor can act directly on Purkinje cells to alter their physiological properties and this fact possibly means that cytokines might be involved in this cellular damage around Purkinje cells, reconfirming the previously proposed role of Purkinje neurons in neuroinflammatory process (Ragagnin et al., 2017).

All in all, the therapeutic approach that has been tested here demonstrated to directly influence neuroinflammatory response. The results provided with this study confirm a limited modulating effect of low dose DEX on the release of immunomodulator peptides from glial cells in a natural sheep scrapie model as well as reaffirm the paradoxical immunomodulator functions that glucocorticoids as DEX exhibit (Munck and Toth, 1992; Filaretova et al., 2009).

**CONCLUSIONS**

This is the first study using a glial-directed strategy in a natural ovine model of scrapie to investigate the contribution of inflammatory responses mediated by cytokines. An impaired communication between microglia and astroglia, which is mediated by cytokines, with IL-1 as

one of the main actors, is suggested here. This study also emphasizes the complex interaction among several pleiotropic neuromodulator peptides and provides a global approach to clarify neuroinflammatory process in natural prion diseases.

The cytokine profiles described in this study suggest the activation of some neurotoxicity-associated pathways. Consequently, targeting such pathways might be a new approach to modify damaging effects of neuroinflammation. Futher studies in order to determine which specific cellular types are expressing these cytokines and how these proteins are involved in cell signalling leading to neurodegeneration are essential.

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# ESTUDIO Nº 4:

## NEUROIMMUNE RESPONSE IN NATURAL PRECLINICAL SCRAPIE AFTER DEXAMETHASONE TREATMENT

# ESTUDIO Nº 4: NEUROIMMUNE RESPONSE IN NATURAL PRECLINICAL SCRAPIE AFTER DEXAMETHASONE TREATMENT

**ABSTRACT**

Chronic dexamethasone treatment in natural scrapie very recently published supported a potential failure of astroglia at advanced stage of disease. Here, to extend the study about the effect of this anti-inflammatory therapy to initial phase of scrapie was intended with the aim of achieving a global approach to the natural neuroinflammatory process occurring in this neurodegenerative disorder. The administration of this glucocorticoid has resulted in an outstanding reduction of vacuolation and aberrant protein deposition (nearly null) and increase of glial activation. Furthermore, evident suppression of IL-1R and IL-6 while exacerbation of IL-1α, IL-2R, IL-10R and IFNγR were also shown. Consequently, the early stage of disease is characterized by an intact neuroglial response similar to that in healthy individuals that tries to re-establish homeostasis. A complex network of neuroinflammatory markers is involved from very early stages of this prion disease which probably turns into impaired at more advances stages. The *in vivo* animal model used here provides essential observations on the pathogenesis of natural scrapie as well as the possibility of establishment of neuroglia as potential target cells by anti-inflammatory therapy.

**Key words:** scrapie, dexamethasone, glucocorticoids, cytokine, neuroinflammation, neuroglia, preclinical stage, prion diseases.

**INTRODUCTION**

Scrapie is the prototype of prion diseases and is one of the most common neurological diseases in sheep, together with coenurosis or leptospirosis, among others (Ligios et al., 2004).

Several reasons have prompted to perform this study about the neuroinflammatory response in naturally affected animals at preclincial stage of scrapie. Firstly, neuroinflammation is an intrinsic concept in neurodegenerative process (Heneka et al., 2014; Ransohoff, 2016) where cytokines have gained huge attention given that they have been demonstrated to be involved in such inflammatory process leading to neurodegeneration (Ransohoff, 2016; Kempuraj et al., 2016; Schwartz and Deczkowska, 2016). Secondly, this complex immune reaction is carried out by neuroglia in this and other neurodegenerative processes. Nevertheless, whether it really plays a neurotoxic (Kordek et al., 1996; Williams et al., 1994, 1997; Gómez-Nicola et al., 2013) or neuroprotective (Zhu et al., 2016; Carroll et al., 2018) role is still undefined. Thirdly, this host response has been demonstrated to be specifically involved in neurodegeneration of prion diseases in sheep (Marcos Carcavilla et al., 2007; Servida et al., 2007; Guijarro et al., 2020), coinciding with the onset of clinical signs in murine model (Campbell et al., 1994) and in CJD (Kordek et al., 1996). Cytokines have been proved to be relevant mediators in the cellular communication in several models of this group of diseases (Campbell et al., 1994; Williams et al., 1994, 1997; Kordek et al., 1996; Veerhuis et al., 2002; Brown et al., 2003; Marcos-Carcavilla et al., 2007).

With this scenario, we recently characterized the glial activation and response in clinical ovine scrapie after chronic anti-inflammatory treatment with dexamethasone (DEX) (Guijarro et al., 2020), demonstrating a potential failure of astroglia at that stage of scrapie. An added value of this *in vivo* model consists of the possibility to extrapolate conclusions drawn from this to other prion (Hernández et al., 2014; Monzón et al., 2018) and prion-*like* diseases (Garcés et al., 2019) in a feasible manner. Studies in murine models are sometimes contradictory and they have not been able to reach a consensus regarding inflammatory response (Perry et al., 2002, Campbell et al., 1994; Williams et al., 1994; Kordek et al., 1996).

All in all, our previous enhancing results about glial response and survival period at clinical stage prompted us to study earlier stages of the disease (Guijarro et al., 2020). Additionally, a previous study has described the presence of several genes involved in inflammation that are up regulated in early phases of prion infection in mice (Carroll et al., 2015). Thus, the specific objective of this study was to assess the effects of this same anti-inflammatory treatment, DEX, in natural scrapie, but at initial phases of disease. By means of description of

neuropathological lesions and glial alterations in themselves and in their communication *via* cytokines released, an approach to a more complete neuroinflammatory profile along the progress of natural scrapie would be achieved. The overall aim is to confirm or discard neuroglia as a potential therapeutic target.

**MATERIAL AND METHODS**

The Ethical Committee of University of Zaragoza approved the following experimental procedures (*Comisión Ética Asesora para la experimentación animal*, 28 Sep 2016. REF: PI41/16). To minimize animal suffering during the experiments and to reduce the number of animals used was intended in all cases.

A total of 12 female Rasa Aragonesa crosses ewes were included in the present study. Two different groups were studied: non-treated (n = 5) and DEX treated preclinical (n = 7) animals. A summary of cases with details about age, genotype for PRNP gene and treatment are shown in Table 1.

**Table 1**. Summary of data corresponding to animals included in the study.

| Sheep Nº | PRNP genotype | Age (years) | Group | Treatment and duration |
|---|---|---|---|---|
| 1 | ARQ/ARQ | 3 | Preclinical | Untreated |
| 2 | ARQ/ARQ | 3 | Preclinical | Untreated |
| 3 | ARQ/ARQ | 6 | Preclinical | Untreated |
| 4 | ARQ/ARQ | 3 | Preclinical | Untreated |
| 5 | ARK/ARQ | 4 | Preclinical | Untreated |
| 6 | ARQ/ARQ | 8 | Preclinical | Treated (7 months) |
| 7 | ARQ/ARQ | 3 | Preclinical | Treated (11 months) |
| 8 | ARQ/ARQ | 7 | Preclinical | Treated (12 months) |
| 9 | ARQ/ARQ | 8 | Preclinical | Treated (13 months) |
| 10 | ARQ/ARQ | 5 | Preclinical | Treated (13 months) |
| 11 | ARQ/ARQ | 5 | Preclinical | Treated (14 months) |
| 12 | ARQ/ARQ | 6 | Preclinical | Treated (17 months) |

These preclinical animals came from monitored flocks belonging to Spanish Scrapie Surveillance Programme and their status was confirmed by the presence of pathological prion protein in recto-anal mucosa associated lymphoid tissue (RAMALT) biopsies (Figures 1A - B). In some few cases, it was necessary to perform Protein Misfolding Cyclic Amplification (PMCA) and Dot Blot (DB) techniques on lymphoid tissues in order to confirm *in vivo* diagnosis (Figure 1C). These animals did no exhibit any clinical signs of scrapie before inclusion in study and throughout the experiment.

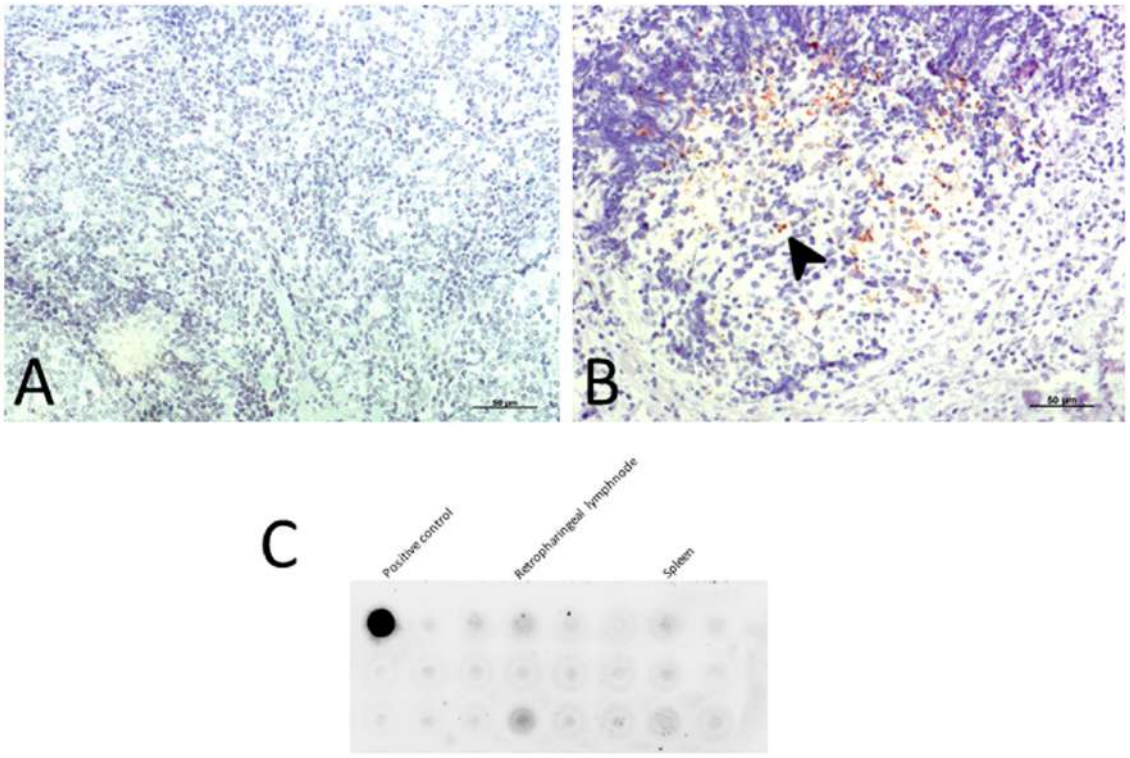


**Figure 1.** *In vivo* diagnosis **(A)** By IHC for $PrP^{sc}$ on biopsies of recto-anal mucosa associated lymphoid tissue (RAMALT) from healthy control and from **(B)** sheep presenting no clinical signs but $PrP^{sc}$ deposits (arrow; preclinical stage of scrapie). Scale bars: 50µm. **(C)** By Dot Blot on a positive retropharyngeal lymph node (product of amplification by PMCA).

Treated preclinical sheep were daily intramuscularly injected with DEX (SYVA, León, Spain; 0.04 mg/kg) in alternating posterior limb after a one-week period of acclimation and until euthanasia by human endpoint criteria due to side effects of DEX (7 months in the shortest and 17 months in the longest case). Additionally, a daily dose of 0.5 mg/kg of omeprazole was administered to all sheep in order to avoid the appearance of gastric ulcers.

Necropsy of each sheep was performed after euthanasia with pentobarbital injection. A total of 80 samples were collected and each one was fixed by immersion in 4% paraformaldehyde for distinct histopathological and immunohistochemical studies. *Postmortem* interval between death and tissue processing was not lesser than 1 hour.

*PROTEIN MISFOLDING CYCLIC AMPLIFICATION (PMCA)*

Samples from lymphoid tissue (spleen or retropharyngeal lymph node) from 5 sheep whose status was doubtful after assessing pathological prion protein in RAMALT biopsies were used for this technique. Tg338 ovinized mice were used to prepare PMCA substrates. PMCA was

performed as previously described (Lacroux et al., 2014; Douet et al., 2017). Briefly, PMCA reactions (50 µL final volume) were seeded with 5 µL of each ovine sample. Then, they were subjected to 3 amplification rounds, each comprising 96 cycles (10 sec sonication, 14 min and 50 sec incubation at 39.5 °C) in a Qsonica700 device. After each round, reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round. PMCA reaction products were analyzed by Dot Blot for the presence of pathological prion protein.

*DOT BLOT (DB)*

Briefly, 18 µl of the product of PMCA from each sample were mixed with proteinase K (18 mg / ml, Roche, Reinach, Switzerland) and incubated for 1 h at 37ºC. Then, 25 µl Laemli (Biorad, France) were added to each sample. After several washes with SDS 1 %, transference to a nitrocellulose membrane was carried out. Membrane was first blocked with PBS - BSA 2% before primary antibody anti- pathological prion protein was added (Sha 31, 1:10.000 for 30 min). After washing, secondary antibody and final revelation were performed.

*HISTOPATHOLOGY (H-E)*

Haematoxyllin - eosin (H-E) staining was applied on paraffin-embedded 4 µm sections in order to visualize the neuropathological lesions in different brain areas: medulla oblongata (MO), obex (O), cerebellum (Cb) and frontal cortex (Fc). As previously published by the group (Monzón et al., 2018; Garcés et al., 2019; Guijarro et al., 2020), neuronal vacuolation and spongiosis were assessed by counting the number of vacuoles present in the grey matter from each section and scored from 0 (minimum) to 4 (maximum) by two independent observers.

*IMMUNOHISTOCHEMICAL TECHNIQUES (IHC)*

Immunohistochemistry (IHC) was carried out in order to assess the accumulation of pathological prion protein, astroglial / microglial activation and neuroinflammatory cytokines released by both glial populations in the four brain areas cited (Fc, Cb, O and MO).

After specific pre-treatments for antigen retrieval, IHC protocols by using specific primary antibodies against pathological prion protein and glial markers were applied. EnVision system (DAKO, Glostrup, Denmark) and diaminobenzidine (DAB; DAKO, Glostrup, Denmark) were used as the visualization system and chromogen, respectively. Hematoxylin counterstaining and mounting in DPX was performed on all sections. Table 2 summarizes each primary antibody and protocol used.

**Table 2.** Primary antibodies used for different immunohistochemical techniques and retrieval method applied for each of them.

| Antibody | Antigen | Type | Dilution | Retrieval method | Source |
|---|---|---|---|---|---|
| **L42** | Prion protein | Monoclonal | 1:500 | Formic acid 15 min<br>Proteinase K 15 min<br>Heat treatment 20 min<br>Peroxidase blocking | DAKO |
| **Anti- GFAP** | GFAP | Polyclonal | 1:500 | Peroxidase blocking | DAKO |
| **Anti-IBA-1** | IBA-1 | Polyclonal | 1:1.000 | Heat treatment 20 min<br>Peroxidase blocking | WAKO |
| **IL-1 alpha** | IL-1 | Polyclonal | 1:100 | Autoclave 121ºC (citrate buffer 10%) | ThermoFisher |
| **Anti-IL-1RN** | IL-1R | Polyclonal | 1:100 | Autoclave 121ºC (citrate buffer 10%) | Sigma |
| **IL-2R.1** | IL-2R | Monoclonal | 1:1.000 | PTLink 96ºC | ThermoFisher |
| **8H12** | IL-6 | Monoclonal | 1:20 | Autoclave 121ºC (citrate buffer 10%) | ThermoFisher |
| **OTI1D10** | IL-10R | Monoclonal | 1:250 | PTLink 96ºC | ThermoFisher |
| **Ber-H2** | TNFαR | Monoclonal | Ready to use | PTLink 96ºC | DAKO |
| **IFNGR1** | IFNγR | Polyclonal | 1:200 | Autoclave 121ºC (citrate buffer 10%) | ThermoFisher |

Immunostained sections were scored by two independent operators on a scale ranging from 0 (minimum) to 4 (maximum), as previously described (Monzón et al., 2018; Garcés et al., 2019; Guijarro et al., 2020), regarding the intensity of pathological prion protein accumulation, astroglial and microglial activation. Morphological glial alterations were also evaluated in 10 microscopic fields in each brain region. For neuroinflammatory markers, observers scored the intensity of immunostaining by counting positive cells (Romero-Trevejo et al., 2010) in 5 microscopic fields in each brain region examined.

- Pathological Prion Protein ($PrP^{sc}$) detection

As previously published (Monleón et al., 2004), 98% formic acid immersion for 15 min, proteinase K (4 µg/ml; Roche, Reinach, Switzerland) treatment for 15 min at 37ºC and hydrated heating for 20 min preceded the endogenous peroxidase blocking (DAKO, Glostrup,

Denmark) for 5 min and incubation with monoclonal antibody L42 (1:500, 30 min RT; DAKO, Glostrup, Denmark).

- Glial Fibrillary Acidic Protein (GFAP) detection for astrogliosis

After endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min, slides were incubated with a polyclonal antibody against glial fibrillary acidic protein (GFAP, 1:500, 30 min RT, DAKO, Glostrup, Denmark).

- Ionized Calcium-Binding Adaptor Molecule-1 (IBA-1) detection for microgliosis

Heat treatment during 20 min was necessary before endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min. Afterwards, sections were incubated with a polyclonal antibody against ionized calcium binding adaptor molecule 1 (IBA-1, at 1:1.000; overnight 4ºC; WAKO, USA).

- IL-1α, IL-1R, IL-6 and IFNγR detection

A pre-treatment consisting of hydrated heating at 121ºC in citrate buffer 10% for 20 min preceded the endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min and incubation with different primary antibodies: polyclonal IL-1α (1:100, overnight 4ºC; ThermoFisher Scientific, Waltham, MA, USA), polyclonal IL-1RN (1:100, overnight 4ºC; Sigma, USA), monoclonal 8H12 (1:20, overnight 4ºC; ThermoFisher Scientific, Waltham, MA, USA) or polyclonal IFNGR1 (1:1200, overnight 4ºC; ThermoFisher Scientific, Waltham, MA, USA).

- IL-2R, IL-10R and TNFαR detection

A pre-treatment consisting of hydrated heating at 96ºC in citrate buffer 10% for 20 min preceded the endogenous peroxidase blocking (DAKO, Glostrup, Denmark) for 5 min and incubation with different primary monoclonal antibodies: IL-2R.1 (1:1.000, overnight 4ºC; ThermoFisher Scientific, Waltham, MA, USA), OTI1D10 (1:250, overnight 4ºC; ThermoFisher Scientific, Waltham, MA, USA) or Ber-H2 (ready to use, 30 min RT; DAKO, Glostrup, Denmark).

*STATISTICAL ANALYSIS*

For results provided by IHC techniques, the normality of distribution was first tested with the Kolmogorov-Smirnov test. The non-parametric Mann-Whitney *U* test was performed to assess quantitative differences between treated and non-treated groups.

SPSS software (SPSS Statistics for Windows, Version 17.0) was used for all analyses and significance in all cases was considered at $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. All graphs were performed with GraphPad Prism 6.0. Data presented in Figures are expressed as means and the standard error of the mean (mean +/- SEM).

**RESULTS**

A summary of statistical results is shown in Table 3. Taking into account the high number of markers used in four different brain areas from two different groups, only one case showing evident statistical differences for each marker has been selected for illustration of results.

As mentioned, no preclinical sheep, regardless treatment, presented any of the key clinical signs of scrapie throughout the time of the experiment in any cases.

**Table 3.** Summary of statistical results obtained in this study.

| Marker assessed | DEX Effect | Brain area | Statistical significance |
|---|---|---|---|
| **Vacuolation** | Decrease | O | $*p \leq 0.05$ |
| **$PrP^{sc}$ deposition** | Decrease | O | $**p \leq 0.01$ |
| | | MO | $**p \leq 0.01$ |
| **GFAP** | Increase (with morphological change) | O | $**p \leq 0.01$ |
| | | MO | $**p \leq 0.01$ |
| **IBA-1** | Increase (with morphological change) | Fc | $*p \leq 0.05$ |
| | | Cb | $\# p = 0.08$ |
| | | O | $*p \leq 0.05$ |
| | | MO | $*p \leq 0.05$ |
| **IL-1α** | Increase | Fc | $**p \leq 0.01$ |
| **IL-1R** | Decrease | MO | $*p \leq 0.05$ |
| **IL-2R** | Increase | O | $*p \leq 0.05$ |
| **IL-6** | Decrease | O | $***p \leq 0.001$ |
| | | MO | $***p \leq 0.001$ |
| **IL-10R** | Increase | Fc | $**p \leq 0.01$ |
| | | Cb | $**p \leq 0.01$ |
| | | O | $\# p = 0.07$ |
| | | MO | $*p \leq 0.05$ |
| **TNFαR** | No changes | | $p > 0,05$ |
| **IFNγR** | Increase | Cb | $**p \leq 0.01$ |
| | | O | $**p \leq 0.01$ |
| | | MO | $**p \leq 0.01$ |

*HISTOPATHOLOGICAL FINDINGS (H-E)*

Vacuolation was mainly located in the neuropil in all cases when it was found, although intraneuronal vacuolation was observed in some of them too. As expected, in regards to differences among brain regions, subtle spongiform change was found in Fc and Cb, while it was the most pronounced and severe in O (Figure 2).

A statistical decrease of spongiform change was found associated to DEX treatment in O (*$p$ = 0.019) (Figure 3A).

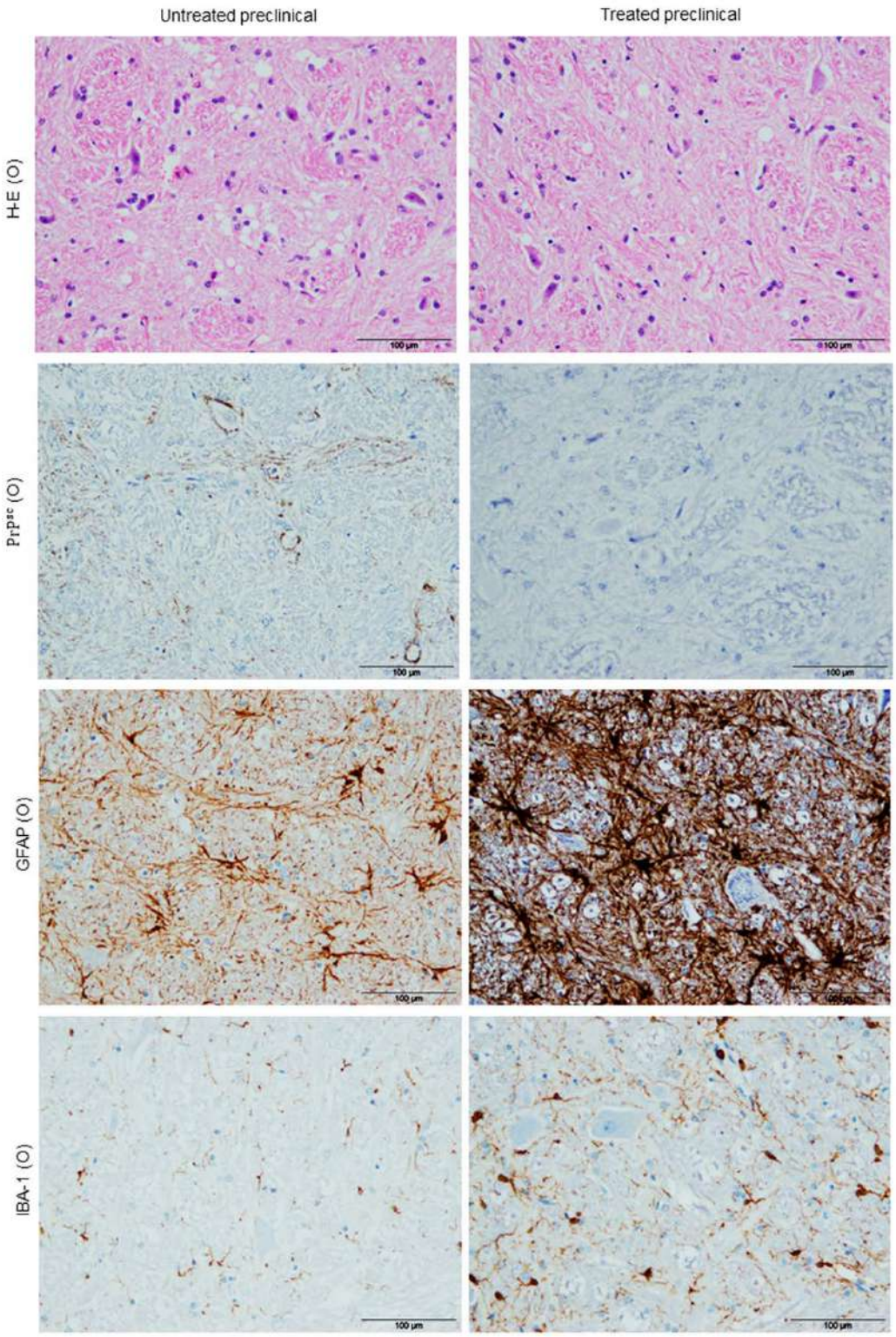


**Figure 2.** Neuropathological findings. DEX treatment in preclinical sheep was associated with a reduction of vacuolation and $PrP^{sc}$ deposits (as illustrated in O), while it was with a huge increase in astro and microgliosis in this same brain area. Scale bars: 100µm.

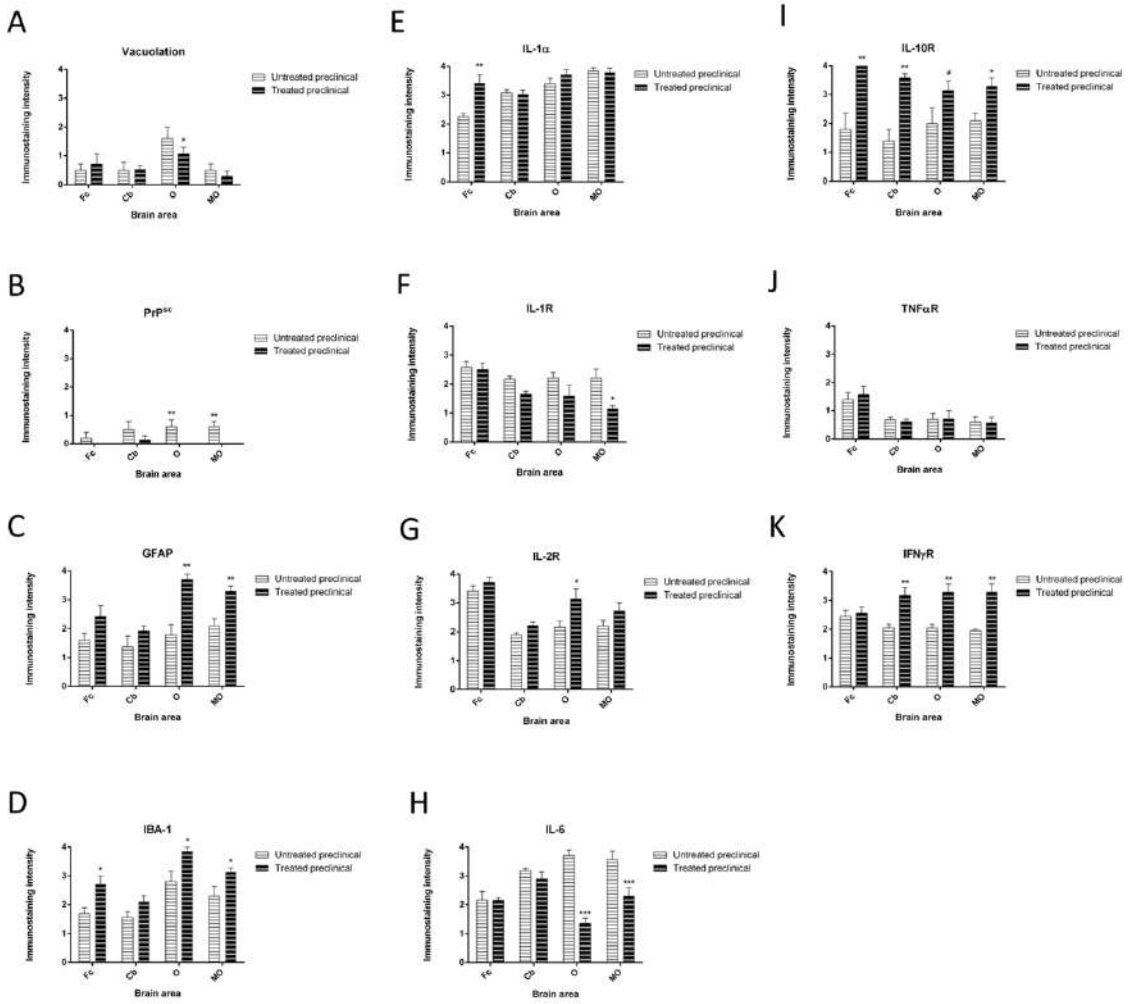


**Figure 3.** Statistical data concerning the variables assessed on the four brain areas (Fc, Cb, O, MO) from DEX treated and non-treated animals **(A)** Vacuolation **(B)** $PrP^{sc}$ deposition **(C)** Astrogliosis **(D)** Microglial proliferation **(E)** IL-1α **(F)** IL-1R **(G)** IL-2R **(H)** IL-6 **(I)** IL-10R **(J)** TNFαR **(K)** IFNγR.

*IMMUNOHISTOCHEMICAL FINDINGS (IHC)*

- *$PrP^{sc}$*

In general, their accumulation was always observed in lymphoid tissues, but not in nervous tissue. Only few $PrP^{sc}$ deposits were observed in brain tissues from some untreated and exclusively in Cb from one treated preclinical animal.

Statistical decrease of their accumulation was evidenced due to treatment in O and MO (** $p$ = 0.004 in both; Figures 2 and 3B).

- *GFAP*

All samples from all brain areas coming from DEX treated sheep showed an evident increase in GFAP immunoreactivity compared with their respective untreated group (strong astrogliosis; Figure 2).

The highest increases of astrogliosis between DEX treated and untreated sheep were observed in O and MO showing very significant statistical differences (**$p$ = 0.008 and **$p$ = 0.004, respectively; Figure 3C).

GFAP immunolabelling showed hypertrophic morphology in treated sheep compared to astrocytes in untreated animals in all brain regions, which appeared more stellate (Figure 4).

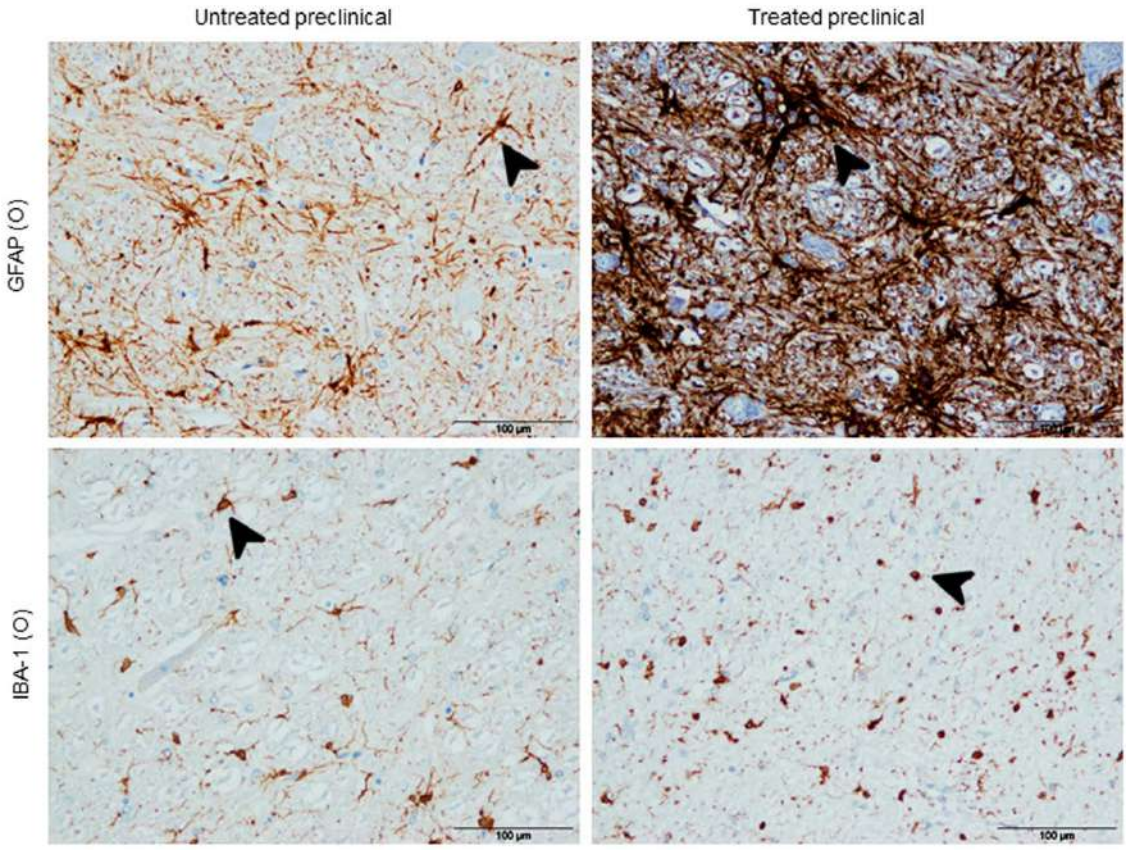


**Figure 4.** Morphological changes in neuroglia associated with treatment. Hypertrophic instead stellate astroglial morphology. And amoeboid instead of ramified microglial morphology (see arrows). Scale bars: 100µm.

- *IBA-1*

IHC for this glial marker always demonstrated an expansion of microglial population in preclinical DEX treated sheep (strong microgliosis) when compared with non-treated animals (Figure 2).

Except for Cb ($p$ = 0.08), all brain regions showed a statistically significant increase (*$p$ = 0,028, *$p$ = 0,035 and *$p$= 0,025) of this glial expansion in DEX treated sheep (for O, MO and Fc, respectively) (Figure 3D).

Regarding morphological changes, a great number of microglial cells showed amoeboid phenotype after DEX treatment in all brain areas, while ramified phenotype was the most frequent in non-treated specimens (Figure 4).

- IL-1

Preclinical sheep showed a very significant increase in IL-1 expression in Fc when they were DEX treated (**$p$ = 0.008; Figure 3E and Figure 5), while no differences were observed in the rest of brain areas analyzed.

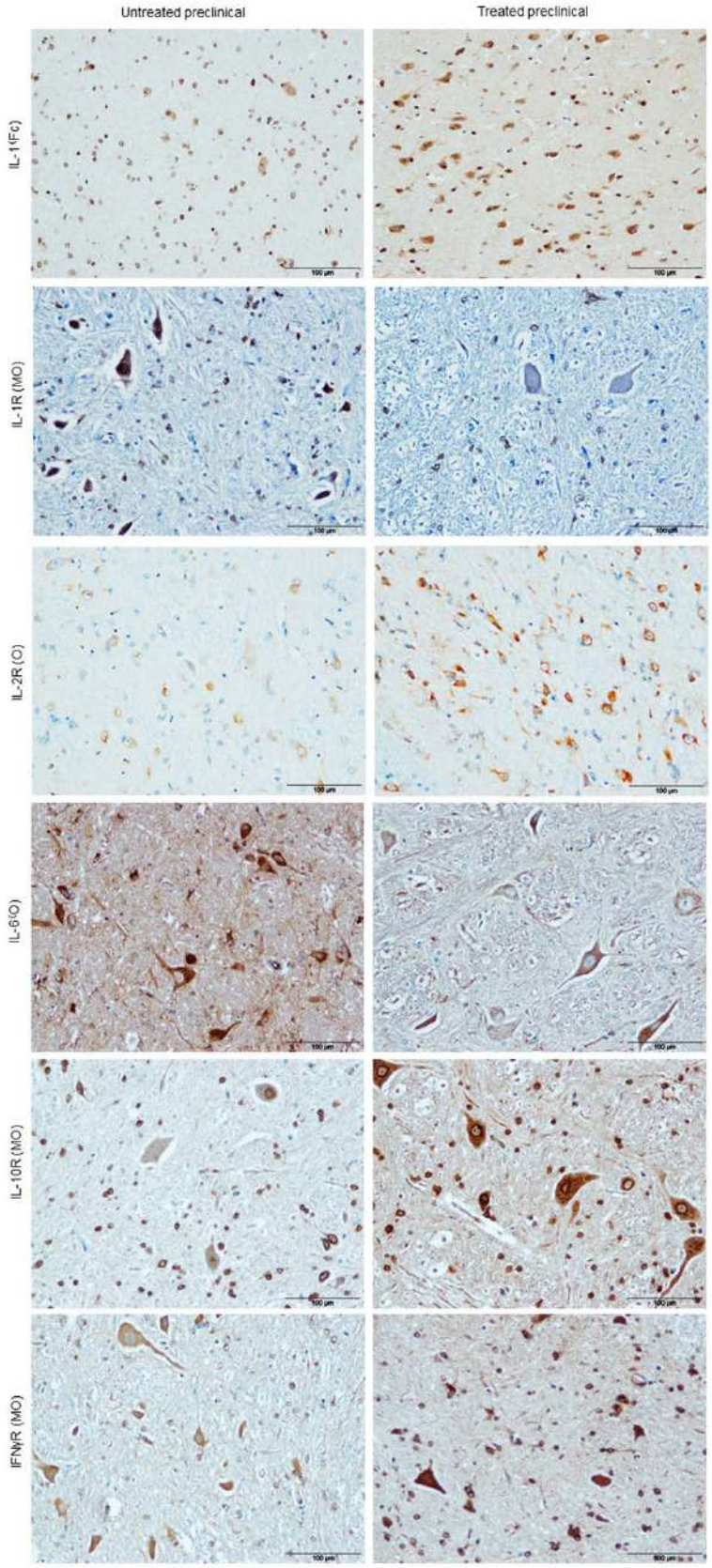


**Figure 5.** Illustration of cases (one area / case per marker) where differences of immunostaining for different cytokines (IL-1α, IL-1R, IL-2R, IL-6, IL-10R and IFNγR) associated with DEX treatment were evident. Scale bars: 100µm.

- IL-1R

Generally, immunostaining for this marker was lower in treated animals compared to the untreated ones, showing statistical significant difference in MO (*$p$ = 0.05; Figure 3F and Figure 5).

- IL-2R

The expression of this receptor increased in all brain areas of preclinical sheep after treatment. Statistically significant difference was only found in O (*$p$ = 0.05; Figure 3G and Figure 5).

- IL-6

Immunostaining for IL-6 in DEX treated sheep was reduced in all brain areas compared to untreated preclinical sheep. This decrease was strikingly evident in O and MO (***$p$ = 0.001 in both areas; Figure 3H and Figure 5).

- IL-10R

Mann Withney *U* test revealed significant effects of DEX in preclinical treated sheep compared to untreated ones, showing an increase of this receptor expression in all brain areas. They were significant in Fc (**$p$ = 0.01) and MO (*$p$ = 0.02), very significant in Cb (**$p$ = 0.003), while a trend was observed in O (#$p$ = 0.07; Figure 3I and Figure 5).

- TNFαR

The immunostaining intensity for this marker was the least intense in all brain areas compared to the rest of neuroinflammatory markers assessed. Moreover, no statistically significant changes in intensity levels were detected for this marker in any brain area after DEX treatment (Figure 3J).

- IFNγR

DEX treated sheep displayed higher intensity for this protein immunostaining compared to the untreated ones in all brain areas (Cb, O and MO; **$p$ = 0.006 in all cases), except for Fc (Figure 3K and Figure 5).

**DISCUSSION**

The findings presented in our recent published study supported an impaired astroglial response at clinical stage of scrapie, suggesting astroglial paralysis (Guijarro et al., 2020). This is in accordance with that recently described in late stages of Alzheimer's disease (AD) (Verkhatsky et al., 2019). A possible impairment of communication between microglia and astroglia at this stage of disease is also suggested (Guijarro et al., 2020) emphasizing the relevance of the complex network of neuromodulator peptides in prion diseases.

Neuropathological and immunohistochemical analyses of neuroinflammatory activity in different brain areas from preclinical sheep naturally infected with scrapie were compared with those DEX treated in the present study. To address this concern, histopathological findings, pathological prion protein accumulation, glial cell activation and several neuroinflammatory markers were investigated. Consistent with the statement that neuroinflammation in prion diseases is region-dependent (Llorens et al., 2014), it has been considered crucial to analyze different encephalic areas: frontal cortex, cerebellum, obex and medulla oblongata.

This work is part of a larger study to get approach to neuroinflammatory process in the neurodegenerative progress in prion diseases. Here, the previous study about the efficacy of the anti-inflammatory therapy on scrapie has been extended to early stages of the disease in order to determine its potential effect in relation with the evolution of neuroinflammatory process along the progress of scrapie. This is based on the fact that mechanisms underlying cytokine release could be a crucial target for therapeutic approaches in central nervous system (CNS) in prion diseases (Garwood et al., 2011). Some other studies have examined very early time points in scrapie but most of them used experimental modes in rodents (Yun et al., 2006; Kretlow et al., 2008; Wang et al., 2019) or in sheep (Halliez et al., 2014), just few of them have used the natural model of the disease (Fernández et al., 2007; Gough et al., 2012; Zetterberg et al., 2019). To study natural field cases of scrapie results, in our opinion, essential because they represent a more feasible source of knowledge than experimental models. Moreover, naturally infected sheep has been demonstrated to constitute a suitable model, not only for prion diseases (Lyahyai et al., 2006), but also for other neurodegenerative disorder research (Monzón et al., 2018; Garcés et al., 2019).

In the present study, it has been demonstrated the efficacy of DEX in reduction of vacuolation and especially in $PrP^{sc}$ deposition, where it becomes practically null in all areas from all treated animals, as main neuropathological lesions associated with the disease. Our previous results in clinical stage of scrapie showed that this treatment was not effective in reducing both vacuolation and prion deposition when neuronal degeneration was advanced (Guijarro et al., 2020). This fact has also been reported in clinical cases of AD treated with non-steroidal anti-inflammatory drugs (NSAIDs) (Imbimbo, 2009), that were ineffective because neuronal degeneration had already started. As consequence, we could speculate that preclinical stage of scrapie constitutes the therapeutic window in which some treatments would be effective in stopping or at least slowing down the progress of neurodegeneration.

Astrocytic marker used in this study evidenced a remarkable increase in all brain areas from all animals after anti-inflammatory treatment. Meanwhile, this same treatment had showed a downregulation of astrogliosis when it was applied at clinical stage of the disease (Guijarro et al., 2020). These observations would reinforce astroglial paralysis in advances stages of scrapie, as recently suggested at late stages of AD (Verkhratsky et al., 2019) while astroglial functions are intact at earlier stages, similar than in healthy subjects.

A relevant astrocytic morphological finding consisting of the presence of hypertrophic astrocytes in treated animals compared to the stellate morphology in non-treated ones is described here, in total agreement with the morphological change undergone by control animals demonstrated in our recent previous study (Guijarro et al., 2020). This supports again the fact that astroglial functions remain similar at preclinical and healthy stages, reacting against any stimulus (treatment in this case). Although changes in astroglial morphology are a recent concept and very scarce information is available (Zeug et al., 2018), this hypertrophic appearance is, to our knowledge, described for first time in preclinical scrapie. Further studies are required in order to determine the real meaning of this and other shape alterations in the progress of neurodegeneration.

In this *in vivo* study, an expansion of the microglial population in DEX treated animals was also proven, showing an increase of the number of microglia as well as an activated and phagocytic amoeboid phenotype. Other authors previously described similar findings (Perry et al., 2002; Perry and O'Connor, 2010; Streit et al., 2014) in association with the expression of neuroinflammatory genes (Kim and de Vellis, 2005). This initial microglial activation might reflect an attempt to start a protective immune response, being activated and expressing more neuroinflammatory genes already at early phases of scrapie infection. With the progress of the

disease, their interglial communication could fail or even a more robust microglial reaction could exacerbate the neurodegenerative process.

Thus, the highest levels of intensity of both microgliosis and astrogliosis were observed in obex and medulla oblongata. Therefore, a protector neuroinflammatory effect is attributed to anti-inflammatory therapy by activation of these both glial populations at preclinical phase of disease. Nevertheless, this drug does not produce effect at advanced stage (clinical animals) probably due to the impaired response (Guijarro et al., 2020).

Overall, variations in all but one of the neuroinflammatory markers assessed in this study have been demonstrated at preclinical stage after treatment.

Preclinical animals showed an increase in IL-1 expression when they were treated. Given that the early overexpression of IL-1 has been fairly related to astrogliosis (Brown et al., 2003; Burwinkel et al., 2004), this fact suggests that secretion of IL-1 by this glial population is properly carried out even at preclinical stage, when communication with microglia is not impaired yet. Moreover, higher levels of this same cytokine was also observed in preclinical studies of AD (Akiyama et al., 2000; Griffin and Mrak, 2002; Sastre et al., 2006), thus reflecting similarities between prion and prion-*like* diseases in neuroinflammatory process aspects.

Just opposite, the immunostaining for IL-1R was lower in all areas from animals after treatment. The loss of IL-1R1 gene expression has been shown to increase the survival time of mice during infection with scrapie (Schultz et al., 2004). Consequently, a beneficial effect of DEX is reaffirmed here at preclinical stage of scrapie. Nevertheless, it is worth mentioning that the self-renewal of microglia is mediated by this peptide (Bruttger et al., 2015). Thus, DEX could be helping to slow down the progress of disease at earlier stages while contributing to a poor communication between microglia and astroglia populations by prevention of this renewal glial process.

It is well - known that IL-2R is involved in neuronal development (Sarder et al., 1996; Dansokho et al., 2016). Provided that an increase of this mediator has been found in all brain areas of preclinical sheep after treatment, a neuroprotective function for DEX would be proposed here. It could be promoting neuronal development, especially in obex, the most affected region by vacuolation and prion deposition and the brain area where the highest increase of this receptor was shown here. There seems to be an attempt to recover the early neuronal loss at preclinical stage but it probably fails in some point in disease progress to clinical and irreversible stage.

A considerable body of data has been compiled about IL-6, describing that it is a pro-inflammatory cytokine in chronic inflammation models (Yamamoto et al., 2000) producing neurologic disease in mouse (Hafiz and Brown, 2000). Its blocking has been even proposed as a potential treatment against chronic inflammatory diseases (Gabay, 2006). Indeed, after anti-inflammatory treatment, immunostaining for IL-6 was detected here to be reduced in all brain areas compared to untreated preclinical sheep. This reduction was especially evident in obex and medulla oblongata. These both areas are, as justified above, where the highest increase of glial activation and prion protein absence were simultaneously observed, supporting that neuroprotection in these areas was especially notable. Consequently, the anti-inflammatory drug may be speculated to be neuroprotective at this stage. And moreover, the crucial role of this cytokine in prion (Veerhuis et al., 2002; Brown et al., 2003; Marcos-Carcavilla et al., 2007) and prion-*like* diseases pathogenesis (Strauss et al., 1992; Wood et al., 1993) is supported by the results provided here.

DEX treatment applied here has shown to produce an outstanding increase of IL-10R in all brain areas of preclinical sheep, which reflects the anti-inflammatory properties of this drug (Couper et al., 2008). As a protecting role has been attributed to this mediator in prion diseases (Thackray et al., 2004; Tamguney et al., 2008), it might be hypothesized that anti-inflammatories could represent a therapeutic target at preclinical stage, despite not when clinical signs have already started.

On the other hand, DEX treatment produced a huge effect on IFNγR expression in preclinical sheep. Taking into account that IFNγ stimulates microgliosis and microglial division (Kim and de Vellis, 2005), it suggests again a neuroprotector role for DEX. Nevertheless, it has been also described that it can lead to neuronal and glial cell damage (Corbin et al., 1996), possibly underlying mechanisms of neurodegeneration at later advanced stages of disease. This hypothesis concurs with the initial activation of microglia observed at preclinical stage, reflecting an attempt of protective response but exacerbating neurodegeneration at clinical stage, possibly mediated by this cytokine.

No changes in levels of TNFαR were detected in any brain area after DEX treatment in preclinical sheep. Furthermore, its immunostaining pattern was the less intense in all brain regions compared to the rest of neuroinflammatory markers assessed in this study. Consequently, a specific role of this mediator in scrapie progress is discarded here, as other authors suggested (Mabbott et al., 2000).

Overall, in the present study the glucocorticoid administered has resulted in a clear suppression of IL-1R and IL-6. Chronic exposition to this drug had been demonstrated to result in suppression of inflammatory cytokines; however, it is worth mentioning that this therapy can also potentiate such immunity (Bowers et al., 2008), especially in CNS (Sorrells et al., 2009). Thus, exacerbation of intensity levels of IL-1, IL-2R, IL-10R and IFNγR has been observed here after chronic exposition to DEX, as it had been described in murine cases of AD treated with DEX (Hu et al., 2016). Actually, it has been described that immune response in prion diseases differ from typical innate immune responses, given that fewer cytokines are elevated and the levels are lower than in conventional CNS infections (Tribouillard-Tanvier et al., 2009, 2012). Therefore, on the basis of the results provided here, it is suggested that the early scrapie infection is characterized by an intact neuroglial response that tries to re-establish homeostasis although it probably turns into impaired at more advances stages of disease.

The *in vivo* animal model used here has provided fundamental observations on the pathogenesis of natural preclinical scrapie, confirming that a complex network of neuroinflammatory markers is involved in the neurodegenerative process since very early stages of infection. Results presented here show that the cytokine network in the encephalon of scrapie affected sheep varies with the progress of the disease and the complexity of this process needs further studies for clarification.

**CONCLUSIONS**

Glucocorticoid treatment applied here directly influences neuroglial response in preclinical sheep naturally affected by scrapie, suggesting that this initial stage of disease could constitute a therapeutic window that is not available at clinical stage, when animals present an impaired astroglial response and irreversible neurodegeneration.

This neuroglia - mediated immune response confirms the occurrence of neuroinflammation in neurodegeneration starting from very early stages of scrapie field infection. It seems that this system is a defensive mechanism displayed by the whole encephalon in pathological conditions, producing an early attempt to re-establish homeostasis but finally failing in advances stages of the disease.

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# VI. CONCLUSIONES

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1. Los resultados obtenidos en esta tesis doctoral demuestran la implicación de una compleja red de citoquinas en el progreso del scrapie natural ovino, apoyando la hipótesis sobre la existencia de una respuesta neuroinflamatoria en el progreso de la neurodegeneración propio de las enfermedades priónicas.

2. Los estudios desarrollados sobre la eficacia de la dexametasona no han logrado demostrar efectos beneficiosos frente al progreso del scrapie cuando los animales presentan un estado avanzado de la enfermedad al haber iniciado ya el proceso de neurodegeneración. Sin embargo, se han obtenido resultados esperanzadores al alargar el periodo de supervivencia en una de las ovejas clínicas tratadas, abriendo así la posibilidad de que estos fármacos puedan tener cierto potencial terapéutico, al menos en algunos casos clínicos.

3. La terapia antiinflamatoria consiguió reducir la vacuolización y especialmente el depósito de proteína prión patológica (anulándolo en todos los animales excepto en uno) en ovejas en estadios tempranos de la enfermedad de scrapie. Por ello, se puede deducir que la modulación de la neuroinflamación mediante fármacos antiinflamatorios podría convertirse en una línea de investigación como potencial diana terapéutica en fases tempranas de las enfermedades priónicas, de forma similar a la que estos fármacos han sido considerados en las enfermedades prion-*like.*

4. El tratamiento administrado influye directamente sobre la respuesta astroglial, tanto en los animales control como en los que se encuentran en estadios preclínicos, mientras que no sucede así en los que están ya en fase clínica de la enfermedad. Estas observaciones sugieren la existencia de una respuesta astrocítica dañada en fases más avanzadas, probablemente relacionada con una posible parálisis astroglial. A pesar de que esta población glial intenta compensar el daño asociado a la acumulación de proteína patológica a través de su proliferación durante los estadios tempranos para restablecer la homeostasis del encéfalo, en algún punto del progreso neurodegenerativo existe un error que bloquea este objetivo final.

5. Se observan esencialmente dos cambios morfológicos neurogliales asociados al tratamiento administrado, tanto en animales control como en los que se encuentran en fase preclínica de scrapie. En el caso de los astrocitos, adquieren una morfología hipertrófica que no había sido previamente descrita. En el caso de la microglía, se observa una prevalencia de la morfología ameboide que parece reflejar la activación de su función fagocítica en fases tempranas del proceso neurodegenerativo.

6. De acuerdo con los perfiles de citoquinas *in situ* descritos en los estudios incluidos en esta tesis doctoral, los patrones de neuroinflamación en scrapie confirman una relación área-dependiente, resultando afectados en mayor medida los tejidos más caudales, como óbex o médula oblongada. Las mayores variaciones observadas tras aplicar el tratamiento consisten en el aumento en los niveles de inmunotinción de los marcadores IL-2R, IL-10R e IFNγR durante el estadio preclínico y la reducción de la expresión de IL-6 independientemente de la fase de la enfermedad.

7. Los resultados obtenidos en el estudio sobre la valoración del tratamiento en estadios clínicos de la enfermedad sugieren la existencia de un fallo de comunicación entre las poblaciones microglial y astroglial, evidenciando la compleja interacción que existe entre dichas poblaciones gliales, mediada por citoquinas liberadas por ambas, otorgando un papel esencial a la IL-1.

8. La expresión de todas las citoquinas y receptores probados resultó mayor en las células de Purkinje que en el resto de células del cerebelo, y también en los animales afectados de scrapie en relación con los animales sanos utilizados como control, reafirmando así el papel crucial propuesto para estas neuronas en el proceso neuroinflamatorio.

9. Se ha desarrollado un modelo de tratamiento crónico de glucocorticoides en la especie ovina capaz de cruzar la barrera hematoencefálica y en el que la afectación cutánea es el principal efecto secundario asociado a su aplicación en esta especie.

10. Una de las principales conclusiones de esta tesis es que se pone en evidencia la importancia de utilizar modelos naturales de la enfermedad, ya que, aunque conllevan dificultades adicionales, reflejan de forma fiable los procesos biológicos que ocurren, en este caso, durante la neurodegeneración.

# VII.CONCLUSIONS

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1. The results provided in this doctoral thesis demonstrate the involvement of a complex network of cytokines in the progress of natural ovine scrapie, supporting the hypothesis about the existence of a neuroinflammatory response in the progress of neurodegeneration characteristic of prion diseases.

2. The studies carried out on the efficacy of dexamethasone have not been able to demonstrate beneficial effects in the progress of scrapie when animals display an advanced stage of the disease and neurodegeneration process has already started. However, encouraging results have been obtained by lengthening the survival period in one of the treated clinical sheep, thus opening up the possibility that these drugs may have some therapeutic potential, at least in some clinical cases.

3. Anti-inflammatory therapy was able to reduce vacuolation and especially pathological prion protein deposition (it managed to annul it in all animals but one) in sheep at early stages of scrapie. Therefore, it can be concluded that modulation of neuroinflammation by anti-inflammatory drugs could become a line of research as a potential therapeutic target at early stages of prion diseases, in a similar manner that these drugs have been considered in prion-*like* diseases.

4. The treatment administered directly influences the astroglial response, both in control animals and in those that are at preclinical stages, whereas this is not the case for those that are already at clinical phase of the disease. These observations suggest the existence of an impaired astrocytic response in advanced stages, probably related to a possible astroglial paralysis. Although this glial population attempts to compensate for the damage associated with the accumulation of pathological protein through its proliferation during the early stages in order to restore brain homeostasis, there is an error at some point in neurodegenerative progress that blocks this final objective.

5. Two neuroglial morphological changes are essentially observed associated with the administered treatment, both in control animals and in those at preclinical phase of scrapie. In the case of astrocytes, they acquire a hypertrophic morphology that had been not previously described. In the case of microglia, a prevalence of amoeboid morphology is observed, which seems to reflect the activation of its phagocytic function at the early stages of the neurodegenerative process.

6. According with the *in situ* cytokine profiles described in the studies included in this doctoral thesis, the neuroinflammation profile confirm a region-dependent relationship in scrapie, being the most caudal tissues, such as obex or medulla oblongata, most affected. The greatest variations observed after applying the treatment consist of the increase in the immunostaining levels of IL-2R, IL-10R and IFNγR markers during the preclinical stage and the reduction of IL-6 expression regardless of the disease stage.

7. The results provided in the study about the evaluation of treatment at clinical stages suggest the existence of a failure in communication between microglial and astroglial populations, showing the complex interaction between such glial populations, mediated by cytokines released by both, giving to IL-1 an essential role.

8. The expression of all cytokines and receptors tested here was higher in Purkinje cells than in other cells in cerebellum, and also in the animals affected by scrapie in relation to healthy controls, thus reaffirming the crucial role proposed for these neurons in the neuroinflammatory process.

9. It has been developed a model of chronic glucocorticoid treatment in ovine species which has been capable of crossing the blood-brain barrier and in which skin lesions are the main side effect associated with its administration in this species.

10. One of the main conclusions of this thesis is that the importance of using natural models of the disease is emphasized, since, although it entails additional difficulties, it reliably reflects the biological processes that occur, in this case, during neurodegeneration.

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