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Alicia Otero García

Estudio de los mecanismos implicados en la barrera de transmisión y en la patogenia de las enfermedades priónicas

Departamento
Patología Animal

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Tesis Doctoral

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Estudio de los mecanismos implicados en la barrera de transmisión y en la patogenia de las enfermedades priónicas

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Facultad de Veterinaria

2018

Centro de Encefalopatías y Enfermedades Transmisibles Emergentes

Departamento de Patología Animal

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ESTUDIO DE LOS MECANISMOS IMPLICADOS EN LA BARRERA DE TRANSMISIÓN Y EN LA PATOGENIA DE LAS ENFERMEDADES PRIÓNICAS

Memoria de tesis presentada por la licenciada

Alicia Otero García

Para optar al grado de Doctora por la Universidad de Zaragoza

Directores:

Dra. Rosa Mª Bolea Bailo

Dr. Juan José Badiola Díez

Zaragoza, junio 2018

Departamento de Patología Animal
Universidad Zaragoza

Centro de Encefalopatías y Enfermedades Transmisibles Emergentes
Universidad Zaragoza

Dña. ROSA Mª BOLEA BAILO y **D. JUAN JOSÉ BADIOLA DÍEZ**, Profesora Titular y Catedrático del Departamento de Patología Animal de la Facultad de Veterinaria de la Universidad de Zaragoza.

INFORMAN:

Que Dña. Alicia Otero García ha realizado, bajo nuestra dirección, los trabajos correspondientes a su Tesis Doctoral titulada "**ESTUDIO DE LOS MECANISMOS IMPLICADOS EN LA BARRERA DE TRANSMISIÓN Y EN LA PATOGENIA DE LAS ENFERMEDADES PRIÓNICAS**" que se corresponde con el plan de investigación aprobado por la Comisión de Doctorado y cumple con los requisitos exigidos por la legislación vigente para optar al Grado de Doctora por la Universidad de Zaragoza, por lo que autorizamos su presentación para que pueda ser juzgado por el Tribunal correspondiente.

En Zaragoza, junio de 2018.

Fdo: Rosa Mª Bolea Bailo

Fdo: Juan José Badiola Díez

Este trabajo ha sido posible realizarlo gracias a un contrato predoctoral del Gobierno de Aragón (C020/2014), cofinanciado por el Fondo Social Europeo, y gracias a la Universidad de Zaragoza, que ha aportado las instalaciones y a la cual pertenecen los Directores del trabajo. Asimismo, ha contado con el 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 de Cooperación Transpirenaica en Seguridad de los Alimentos de Origen Ovino y Caprino (ConCOTSA EFA 205/11), Proyecto de Consolidación de la Cooperación Transpirenaica en materia de Seguridad Alimentaria EFA 282/13 (Transprion) y Red de Investigación Transfronteriza en Enfermedades Priónicas Humanas y Animales EFA 148/16 (Redprion)

-Proyecto AGL2015-65560-R: Implicación de la glicosilación en la Transmisión de las Enfermedades Priónicas, Biomarcadores Moleculares y Papel de las Neurotrofinas en la Neurodegeneración, del Ministerio de Economía y Competitividad.

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Universidad Zaragoza

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Dña. ROSA Mª BOLEA BAILO y **D. JUAN JOSÉ BADIOLA DÍEZ**, Profesora Titular y Catedrático del Departamento de Patología Animal de la Facultad de Veterinaria de la Universidad de Zaragoza.

INFORMAN:

Que Dña. Alicia Otero García bajo nuestra dirección, presenta el trabajo correspondiente a su Tesis Doctoral titulada "**ESTUDIO DE LOS MECANISMOS IMPLICADOS EN LA BARRERA DE TRANSMISIÓN Y EN LA PATOGENIA DE LAS ENFERMEDADES PRIÓNICAS**" 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:

Dr. Hicham Filali, IM2A (Institut de la Mémoire et de la Maladie d´Alzheimer), Bâtiment François Lhermitte, Hôpital de la Salpêtrière, 47/83 Boulevard de l´hôpital, 75013 Paris, France. Doctor por la Universidad de Zaragoza el 30/07/2013

Dr. Jaber Lyahyai, Professeur Assistant en Génétique, Faculté de Médecine et de Pharmacie, Departement of Medical Genetics, Université Mohammed V, Impasse Souissi, Rabat 10100, Rabat, Morocco. Doctor por la Universidad de Zaragoza el 21/06/2007

Asimismo, avalamos la realización por parte de la doctoranda de una estancia en una institución de investigación extranjera durante tres meses:
Estancia desde 15 de septiembre hasta el 20 de diciembre de 2016 (96 días) en el Centre for Prions and Protein Folding Diseases (CPPFD), University of Alberta, Edmonton, Alberta, Canada, bajo la supervisión de la Dra. Debbie McKenzie, en la que la estudiante de doctorado trabajó en el uso de técnicas de infección de cultivos celulares y su aplicabilidad a la investigación de las enfermedades priónicas, así como en estudios

relacionados con la patogenia de la enfermedad crónica caquectizante de los cérvidos. Por tanto, cumple con los requisitos necesarios para optar al Título de Doctora con Mención Internacional.

En Zaragoza, junio de 2018.

Fdo: Rosa Mª Bolea Bailo

Fdo: Juan José Badiola Díez

Universidad Zaragoza
1542

SR. PRESIDENTE DE LA COMISIÓN DE DOCTORADO
UNIVERSIDAD DE ZARAGOZA

INFORME VALORACIÓN TESIS DOCTORAL / PhD THESIS ASSESSMENT REPORT

Doctorando-a /Doctoral Student: ALICIA OTERO GARCÍA

Título de la Tesis / Title of the Thesis: ESTUDIO DE LOS MECANISMOS IMPLICADOS EN LA BARRERA DE TRANSMISIÓN Y EN LA PATOGENIA DE LAS ENFERMEDADES PRIÓNICAS

D./~~Dª~~. HICHAM FILALI

☐ 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 Paris, a 04 de Junio de 2018

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).

I have read with interest the scientific work of Ms. Alicia Otero entitled "estudio de los mecanismos implicados en la barrera de transmisión y en la patogenia de las enfermedades priónicas". The subject of this thesis is of great importance in the field of TSE agent transmission and pathogenicity. This thesis is presented as a compendium of five scientific papers of which one has already been published in prestigious Journal (*Molecular Neurobiology*) and four are presented as preliminary manuscripts. The findings of the first three works contribute to a good understanding of TSE agent transmission, while the last two ones describes some of molecular pathogenicity mechanisms of prion protein. It is important to emphasis that these two aspects of TSE field are of great scientific importance because they still very unclear.

Ms. Alicia addressed 40 pages of well chosen topics in the chapter of the bibliographic review where she collected the last scientific data to set up the scene for the work undertaken in this thesis.

In the first publication, the PhD candidate tested the specific substitution of a key amino acid characteristic of canids N158D on the conversion of PrP^{C} to PrP^{Sc} when co-expressed with murine wild type PrP^{C}. The results demonstrate that after the inoculation of different prion strains (22L, ME7, RML, or 301C), mice expressing N158D PrP showed a significant increase in survival times (by 45% to 113%) in comparison with the wild type mice, without any observed strain modification. This study suggest that this substitution confers dominant-negative effect on prion protein preventing PrP^{Sc} formation and delaying the disease.

In the same direction, the second study investigated the effect of N158D PrP substitution on survival period of mice overexpressing bank vole I109 PrP^{C}, a highly susceptible model to a wide range of TSEs, after inoculation with two prion isolates of different characteristics. Again, those transgenic mice showed longer survival periods (from 52 to 108%), confirming the protective effect of the N>D amino acid substitution against the PrP^{Sc} propagation even in highly susceptible model.

The previous two studies show that N>D prion protein amino acid substitution could have an important use to develop new therapeutic approaches against TSEs.

The third paper aimed mainly to evaluate the effect of prion protein glycosylation in the interspecies prion transmission. Thus, TgNN6h, a murine model expressing the non-glycosylated human PrP^{C} was challenged by many prion isolates. The results showed that only BSE-related isolates has the capability to cross the human transmission barrier for TSEs in TgNN6h. suggesting that the absence of glycans in human prion protein does not affect the pathological characteristic of the prion isolates used in this study.

After these three studies focused on TSE agents transmission, the following two ones investigated some of molecular pathogenicity mechanisms of prion protein.

The fourth paper evaluated the impairment of the ubiquitin-proteasome system and the endoplasmic reticulum stress in brain samples from murine models of spontaneous TSE (TgVole mice). The results showed that events related to those two aspect of pathogenicity are only observed in clinical mice, indicating that these events could not be considered as key pathogenic mechanisms during spontaneous TSE.

In the last study, the PhD candidate assessed the distribution pattern of PrP^{CWD} in numerous tissues of white-tailed deer of different *Prnp* genotypes and orally challenged with Wisc-1 prion strain. This work concluded that in accordance with previous works, resistant polymorphism in CWD could limit PrP^{CWD} propagation in many tissues.

Overall, this Doctoral Thesis reflects the ability of Ms. Otero for a high quality and independent scientific research work. The topic is of enormous current significance, the research approaches are systematic, and the results provide important new advances in the fields of TSE transmission and pathogenicity in small ruminants.

In conclusion, I recommend strongly this work to be submitted for examination as a Doctoral Thesis.

I also would like to congratulate Ms. Otero for her high-quality research, and I wish her the very best of success for the immediate future.

June 4th, 2018

Yours sincerely,

Hicham Filali, PhD
IM2A (Institut de la Mémoire et de la Maladie d'Alzheimer)
Bâtiment François Lhermitte
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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: ..... Alicia OTERO.....

Título de la Tesis / Title of the Thesis:

Estudio de los mecanismos implicados en la barrera de transmisión y en la patogenia de las enfermedades priónicas

D./Dª. .....Jaber LYAHYAI.....

☐ 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

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En Rabat, a 06 de Junio de 2018..

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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).

Firstly, I'm thankful for the invitation to review the Ph.D. thesis of **Ms. Alicia OTERO**.

The submitted thesis entitled "**Estudio de los mecanismos implicados en la barrera de transmisión y en la patogenia de las enfermedades priónicas**" contributes to the current knowledge of certain mechanisms related to the transmission barrier for Transmissible Spongiform Encephalopathies and their pathogenesis.

This PhD thesis is well structured and correctly presented. It is written on 190 pages altogether, and enriched by number of figures (18) and tables (4).

The thesis consists of five chapters, beginning with the presentation and the aim of thesis as the first part of thesis, the second part is an interesting bibliography review (60 pages). The results are presented in the form of five scientific studies of which one has already been published in a highly ranked journal "**Molecular Neurobiology**" and four are presented as preliminary manuscript for main article. The two last chapters contain a general discussion and conclusions of the thesis.

The structure of thesis conforms to principles and requests to the structure of scientific thesis.

The first Paper investigated the impact of amino acid change at codon 158 of (Asparagine (N) by aspartic acid (D)) in the murine PrP^{C}. The results show that this substitution acts as a dominant-negative protein to partially block the conversion of PrP^{C} to PrP^{Sc} and delaying disease onset without altering the neuropathological properties of the prion strains.

The second Manuscript investigated if whether the substitution N159D could also prevent or delay the onset of the prion disease in a highly susceptible model. The results agree with the previous research, demonstrating that the expression of this specific amino acid change in PrP is able to confer a protective effect against the propagation of notably different strains, even when expressed in a PrP^{C} highly susceptible to misfolding.

The third study looked at whether the absence of glycans in the human PrP^C could affect the transmission barrier and deformation characteristics of BSE prions. The findings suggest that the glycosylation of human PrP^C is not essential for the preservation of the human transmission barrier for prions or for the maintenance of BSE strain properties.

The fourth study investigated the role of Endoplasmic Reticulum (ER) stress and proteasome impairment during the course of spontaneous prion disorders in a murine model. The results show that ER stress and Ubiquitin-Proteasome System (UPS) dysfunction seem to be collateral events associated with prion neuropathology rather than essential mechanisms in the pathogenesis of sporadic prion diseases.

And the last Manuscript indicates that the expression of Chronic Wasting Disease resistance-associated polymorphisms, as observed in other prion diseases, is able to limit the distribution of PrP^{CWD} aggregates in a wide variety of tissues and supports previous findings on the importance of the deer PRNP genotype on the modulation and adaptation of CWD strains.

Finally, the author has studied and used appropriate number of bibliography sources used and quoted in the thesis. It is the evidence of the deep theoretical knowledge and very good orientation in the discussion of the thesis findings.

Overall, the reviewed thesis presents original research results of large importance and it fulfills all requirements aimed for obtaining PhD degree. This thesis is ready to be defended orally, in front of respective committee.

Sincerely yours,

06th June 8, 2018

Pr. Jaber LYAHYAI
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Universidad Zaragoza
1542

CERTIFICADO DE ESTANCIA DE INVESTIGACIÓN

El abajo firmante, director del departamento de ..... de la Universidad de Zaragoza o coordinador del programa de doctorado en Medicina y Sanidad Animal ..... de la Universidad de Zaragoza (elíjase una de las dos opciones),

CERTIFICA que

D/D.ª Alicia Otero Garcia ....., procedente de la Universidad de Zaragoza ..... ha realizado desde el 15-Sep-2016 .....*(fecha de inicio)* hasta el 20-Dec-2016 .....*(fecha final)*

una estancia de investigación enmarcada en el desarrollo de su tesis doctoral en el departamento/instituto/centro de Centre for Prions and Protein Folding Dise (identifíquese lo que proceda).

Centre for Prions and Protein Folding Diseases

Y para que así conste, firmo la presente

en Zaragoza, a de de 2015

Fdo.: ..... ☐ El director del Departamento ☐ El coordinador del programa de doctorado	Visto bueno del responsable/tutor Durante la estancia Fdo.

A mi familia,

José Antonio, Alicia y Andrés

Try not to become a person of success, but rather try to become a person of value

Albert Einstein

LISTA DE ABREVIATURAS

µl. Microlitro

µg. Microgramo

ºC. Grado centígrado

g. Gramo

ml. Mililitro

kg: kilogramo

BiP. Proteína de unión a inmunoglobulina.

BSE. Bovine Spongiform Encephalopathy.

CA. Caudate nucleus

Cbl. Cerebellar cortex.

Cc. Corpus callosum.

Cgl. Cerebellar granular layer

CJD. Creutzfeldt-Jakob disease.

Cml. Cerebellar molecular layer

Cpl. Cerebellar Purkinje cell layer

CNS. Central nervous system.

CWD. Chronic Wasting Disease.

CWD-vole. Cepa de CWD adaptada al *bank vole*

Cwm. Cerebellar white matter

DAB. Diaminobenzidine.

Dpi. Days post-inoculation

Dpl. Proteína doppel

ECC. Enfermedad crónica caquectizante.

ECJ. Enfermedad de Creutzfeldt-Jakob.

EEB. Encefalopatía espongiforme bovina.

eECJ. Enfermedad de Creutzfeldt-Jakob esporádica.

EEF. Encefalopatía espongiforme felina.

EET. Encefalopatías espongiformes transmisibles.

ER. Endoplasmic reticulum.

ERAD ER-associated degradation.

ETV. Encefalopatía transmisible del visón.

Fc. Frontal cortex.

FCgm. Frontal cortex grey matter

FCwm. Frontal cortex white matter

fECJ. Enfermedad de Creutzfeldt-Jakob familiar.

GFAP. Glial fibrillary acidic protein.

GFP. Green fluorescent protein.

GPI. Glicosilfosfatidilinositol.

GSS. Enfermedad de Gerstmann-Sträussler-Scheinker.

Hc. Hippocampus

Hsp70. Heat stock proteins

Ht. Hipothalamus

IEF. Insomnio esporádico fatal.

IFF. Insomnio familiar fatal.

IHC. Immunohistochemistry.

IHQ. Immunohistoquímica.

Kb. Kilobases.

kDa. Kilodaltons

mAb. Monoclonal antibody

Mes. Mesencephalon.

Mo. Medulla oblongata.

NA. Not applicable

NAPA. Nonadaptative prion amplification.

Nor98. Forma atípica del scrapie

OB. Olfactory bulb

Oc. Occipital cortex.

PBS. Phosphate buffered saline.

PCR. Polimerase chain reaction.

PDI. Proteína disulfuro-isomerasa.

PIRIBS. Parallel In Register Intermolecular β-Sheet.

PK. Proteinasa K

PMCA. Amplificación cíclica de proteínas mal plegadas.

PPI. Peptidil-prolil cis-trans isomerasas

PRND. Gen que codifica la proteína doppel

PRNP. Gen que codifica la proteína prion

PrP. Proteína prion

PrP 27-30. PrP resistente a PK de 27-30 kDa

PrP^{C}. Proteína prion celular

PrP^{res}. PrP resistente a la digestión con proteasa

PrP^{Sc}. Proteína prion patológica

PVSPr. Prionopatía variable sensible a la proteasa.

RE. Retículo endoplásmico.

RER. Retículo endoplasmático rugoso.

RMN. Espectroscopía mediante resonancia magnética nuclear.

Sa. Septal area.

SC. Superior colliculus

sCJD. Sporadic Creutzfeldt-Jakob disease.

SEM. Standard Error of the Mean

Sho. Proteína shadoo

SLR. Sistema linforreticular.

SN. Septal nucleus

SNC. Sistema nervioso central.

SNP. Sistema nervioso periférico.

SPRN: Gen que codifica la proteína shadoo

SSBP/1. Scrapie subpassaged brain pool 1.

T. Thalamus.

TBS. Tris buffered saline

Tc. Thalamic cortex.

TSE. Transmissible Spongiform Encephalopathies.

Ub^{G76V}-GFP. Ubiquitina marcada con GFP

UPR. Unfolded protein response.

UPS. Sistema ubiquitino-proteasómico./*Ubiqutin-proteasome system*

vCJD. variant Creutzfeldt-Jakob disease.

vECJ. variante de la Enfermedad de Creutzfeldt-Jakob.

Wt. Wild-type

Aminoácidos

A: Alanina	H: Histidina	N: Asparagina	T: Treonina
C: Cisteína	I: Isoleucina	P: Prolina	V: Valina
E: Ácido glutámico	K: Lisina	Q: Glutamina	Y: Tirosina
F: Fenilalanina	L: Leucina	R: Arginina	W: Triptófano
G: Glicina	M: Metionina	S: Serina	

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ANTECEDENTES Y OBJETIVOS

Las enfermedades priónicas o encefalopatías espongiformes transmisibles (EET) constituyen un conjunto de enfermedades neurodegenerativas fatales de los animales y los humanos. Estos procesos cursan con largos periodos de incubación y se caracterizan por la degeneración espongiforme del sistema nervioso central (SNC), con la aparición de espongiosis del neuropilo, vacuolización intraneuronal, pérdida neuronal y reacciones neuroinflamatorias de las células gliales. El agente etiológico de estos procesos es una proteína anómala (PrP^{Sc}) que se genera por la conversión conformacional de una glicoproteína codificada de forma fisiológica por el hospedador (PrP^{C}). La secuencia de la PrP^{C} contiene dos sitios susceptibles de ser glicosilados mediante la adición de N-glicanos, lo cual da lugar a tres formas maduras de la PrP^{C}: la forma diglicosilada, monoglicosilada y no glicosilada.

Estas enfermedades pueden aparecer de forma natural en multitud de especies de mamíferos, incluido el hombre; bien sea por causas esporádicas, genéticas o adquiridas. Ejemplos de estos procesos patológicos son el scrapie en ovinos y caprinos, la encefalopatía espongiforme bovina (EEB), la enfermedad crónica caquectizante de los cérvidos (ECC) o la variante de la enfermedad de Creutzfeldt–Jakob en el hombre (vECJ). Esta última se asoció al consumo de tejidos de bovinos infectados con EEB, hecho que tuvo una enorme repercusión en la seguridad alimentaria y en la salud pública. Si bien no se han descrito transmisiones naturales al ser humano de otras enfermedades priónicas animales, sí se ha conseguido cruzar la barrera de transmisión interespecie en ciertos estudios en los que se han utilizado técnicas de propagación in vitro o bioensayos en modelos transgénicos que expresan la PrP^{C} humana. Asimismo, las EET han sido transmitidas de forma experimental a numerosas especies animales en las que nunca se han observado casos naturales (Barria et al., 2014; Cassard et al., 2014). Sin embargo, entre los mamíferos existen tres grupos de animales que durante décadas han sido considerados resistentes o muy poco susceptibles a las EET: los lepóridos, los équidos y los cánidos (Khan et al., 2010). Diversos estudios in vitro e in vivo demostraron que la PrP^{C} del perro es la que muestra una mayor resistencia al malplegamiento, sugiriéndose posteriormente que la expresión de ácido aspártico (D) en el codón 163 de la proteína prion de esta especie podría ser la clave para la inusual resistencia que presentan los cánidos a las enfermedades priónicas (Fernandez-Borges et al., 2017b). En el extremo opuesto de la escala de susceptibilidad a las EET se encuentra el *bank vole* o topillo rojo (Myodes glareolus), una especie extremadamente susceptible a la inoculación de multitud

de cepas priónicas de diversos orígenes y características. Además, en el año 2012, se descubrió que los ratones transgénicos que sobreexpresan la PrP^C del *bank vole* con isoleucina (I) en el codón 109 desarrollan una EET de forma espontánea, hecho que se atribuyó a la gran facilidad para el malplegamiento que presenta la PrP^C de esta especie (Watts et al., 2012). Por ello los ratones transgénicos TgVole I109PrP se convirtieron en un modelo valioso para el estudio de las EET esporádicas o espontáneas, cuyo origen sigue siendo desconocido. En la primera parte de esta tesis se han incluido dos estudios cuyo objetivo es evaluar el posible potencial terapéutico que tendría la introducción de sustituciones aminoacídicas propias de hospedadores muy poco susceptibles a las EET en la PrP^C de otras especies, así como explorar el posible efecto dominante negativo de estas proteínas mutadas.

Las enfermedades priónicas tienen siempre un curso fatal, ya que hasta la fecha no se conocen tratamientos efectivos frente a ellas. Además, todavía existen muchas incógnitas sobre los factores que regulan la barrera de transmisión y la patogenia de estos procesos. Asimismo, existe una gran variedad de cepas priónicas de muy distintas características patobiológicas, lo cual dificulta aún más el conocimiento de los mecanismos patogénicos reguladores de las EET. En 1997 se postuló que la variación en la glicosilación de la PrP^C podría estar relacionada con su conversión en la forma patógena y también con las propiedades de las cepas priónicas transmitidas (DeArmond et al., 1997). Posteriormente, diversos estudios sugirieron que la glicosilación de la proteína prion era un factor regulador fundamental de la barrera de transmisión intra- e interespecie (Tuzi et al., 2008; Wiseman et al., 2015). En el tercer estudio de esta tesis evaluamos cuál es el efecto de la glicosilación de la PrP^C humana en la barrera de transmisión y en las características de diversas cepas priónicas.

Los mecanismos patogénicos implicados en la neurodegeneración asociada a las enfermedades priónicas tampoco se conocen con exactitud. Se ha propuesto que ciertos mecanismos reguladores de la proteostasis, como son el estrés del retículo endoplásmico o la degradación proteica por parte del sistema ubiquitino-proteasómico, podrían jugar un papel en la patogenia, no sólo de las EET, sino de otras enfermedades neurodegenerativas caracterizadas por la acumulación de proteínas anómalas como la enfermedad de Alzheimer, de Párkinson, de Huntington, o la esclerosis lateral amiotrófica (Hetz and Mollereau, 2014). El fallo de estos sistemas para controlar la acumulación de proteínas patógenas podría estar implicado en la neurodegeneración asociada a estas enfermedades.

Por ello, en el cuarto estudio de esta tesis evaluamos marcadores de estrés del retículo endoplásmico y de la disfunción de la degradación proteica del sistema ubiquitino-proteasómico, con el fin de investigar qué papel desempeñan estos mecanismos en el curso de las EET espontáneas.

Por último, analizaremos si ciertos polimorfismos asociados con la resistencia a la ECC, una enfermedad actualmente emergente en Europa, pueden influir en la patogenia de la enfermedad, afectando al fenotipo de la enfermedad y/o a la diseminación de la PrP^{CWD} hacia los tejidos periféricos en ciervos inoculados por vía oral. Este efecto se ha descrito para ciertos polimorfismos de resistencia del gen *PRNP* ovino (Gonzalez et al., 2014b), hecho relevante ya que la selección de animales portadores de estos alelos ha demostrado ser una herramienta muy útil para el control y la erradicación del scrapie.

En esta tesis se han realizado cinco estudios cuyo objetivo general es el de contribuir a un mejor conocimiento de ciertos mecanismos relacionados con la barrera de transmisión para las EET y su patogenia. En el siguiente apartado se describen los objetivos planteados en esta tesis, así como los estudios que se han llevado a cabo para cumplirlos.

El objetivo principal de esta tesis doctoral titulada: **"Estudio de los mecanismos implicados en la barrera de transmisión y en la patogenia de las enfermedades priónicas"** es profundizar en el conocimiento de las encefalopatías espongiformes transmisibles (EET) o enfermedades priónicas, fundamentalmente desde dos puntos de vista. Por un lado, se pretende evaluar qué factores regulan la transmisión intra- e interespecie de los agentes priónicos mediante la realización de experimentos en modelos murinos transgénicos de distinta base genética. Por otro lado, se intentará conocer más detalles sobre los mecanismos reguladores de la patogenia de estos procesos, que no es todavía completamente conocida, estudiando cuál es la implicación del estrés del retículo endoplásmico y la disfunción de la proteolisis mediada por el proteasoma en encéfalos de ratones afectados por una EET espontánea y el efecto de ciertos polimorfismos de resistencia del gen *PRNP* sobre la patogenia de una enfermedad priónica infecciosa como es laECC.

Esta tesis está, por tanto, organizada en cinco estudios con los siguientes objetivos:

Estudio Nº 1: An amino acid substitution found in animals with low susceptibility to prion diseases confers a protective dominant-negative effect in prion-infected transgenic mice (*published in Molecular Neurobiology*).

- Objetivo general: Determinar si el cambio de asparagina (N) por ácido aspártico (D) en el codón 158 de la PrP^{C} murina, un cambio aminoacídico exclusivo de los cánidos, es capaz de prevenir la formación de PrP^{Sc} cuando esta proteína mutada se coexpresa con la PrP^{C} murina de tipo salvaje (*wild-type)*.
- Objetivos específicos:
    - Definir y comparar estadísticamente los periodos de supervivencia de ratones Tga20xN158D, portadores de la sustitución asociada con la resistencia de los cánidos a las EET, con los obtenidos en ratones Tga20xKO inoculados con las mismas cepas cepas priónicas: 3 cepas de scrapie adaptadas al ratón: 22L, ME7 y RML, y una cepa de EEB murina: 301C.
    - Obtener un perfil lesional de estos animales mediante un estudio histopatológico del encéfalo y su patrón de depósito de PrP^{Sc} mediante la técnica de PET Blot.

- Comparar los perfiles neuropatológicos obtenidos en los distintos genotipos con el fin de evaluar si el cambio aminoacídico altera las características de la enfermedad.

Estudio Nº2: A single amino acid substitution, characteristic of mammals poorly susceptible to prion diseases, delays the propagation of different prion strains in highly susceptible transgenic models (*preliminary manuscript for main article*).

- Objetivo general: Determinar si la sustitución N159D de la PrP^{C} es capaz de prevenir la enfermedad o retrasar la aparición de signos clínicos en ratones transgénicos que expresan la PrP^{C} del topillo rojo o *bank vole*, una de las especies más susceptibles a las enfermedades priónicas.
- Objetivos específicos:
  - Definir y comparar estadísticamente los periodos de supervivencia de ratones TgVole-N159D, portadores de la sustitución que nos interesa estudiar, con los obtenidos en ratones TgVole, ambos modelos inoculados con dos cepas priónicas distintas: una cepa clásica (CWD-vole) y una cepa atípica de origen espontáneo (Sp-TgVole).
  - Obtener un perfil lesional de estos animales mediante un estudio histopatológico del encéfalo y su patrón de depósito de PrP^{Sc} mediante la técnica de PET Blot.
  - Comparar los perfiles neuropatológicos obtenidos en estos modelos con el fin de conocer si la presencia de la sustitución aminoacídica ha producido algún cambio en las características de la enfermedad.
  - Comparar los periodos de supervivencia y las características neuropatológicas observadas en los animales inoculados con la cepa Sp-TgVole con los resultados obtenidos en ratones TgVole-N159D y TgVole sin inocular, que desarrollan una EET de forma esponánea.

Estudio Nº3: N-attached glycans are not indispensable for the maintenance of human transmission barrier for prions or the preservation of BSE strain properties (*preliminary manuscript for main article*).

- Objetivo general: Conocer si la glicosilación de la PrP^{C} humana juega un papel importante en la barrera de transmisión del ser humano frente a diversas cepas priónicas. Asimismo, y dado que la EEB sí se transmite a los humanos en

condiciones naturales, se pretende determinar si la ausencia de glicanos en la PrP^C humana puede alterar las características de la EEB cuando esta se transmite al ser humano.

- Objetivos específicos:
    - Inoculación de ratones TgNN6h, que expresan una PrP^C humana no glicosilada, y de ratones Tg340, que expresan una PrP^C humana normalmente glicosilada, con distintas cepas priónicas: SSBP/1 (scrapie clásico), scrapie atípico, ECC, BSE-L y BSE-H (EEB atípicas), BSE-C (EEB clásica), EEB ovina, EEB porcina y vECJ.
    - Definir los signos clínicos, periodos de supervivencia y características neuropatológicas de los animales enfermos, así como caracterizar el perfil de glicosilación de la PrP^{res} mediante la técnica de Western blot.
    - Evaluar la eficiencia de propagación *in vitro* de estas cepas priónicas en sustrato TgNN6h utilizando la técnica de amplificación cíclica de la proteína malplegada (PMCA).
    - Generar *in vitro* cepas priónicas adaptadas al sustrato humano no glicosilado y evaluar su eficiencia de transmisión en ratones TgNN6h.
    - Comprobar si las características neuropatológicas y bioquímicas de las cepas de EEB se mantienen tras su transmisión a ratones que expresan una PrP^C humana no glicosilada.

Estudio Nº4: Spontaneous prion-associated neurodegeneration in transgenic mice causes both ER stress and proteasome impairment at the terminal stage of prion diseases (*preliminary manuscript for main article*).

- Objetivo general: Comprobar si durante el curso de las enfermedades priónicas espontáneas se produce estrés del retículo endoplásmico o un bloqueo de la función del sistema ubiquitino-proteasómico (UPS) utilizando muestras de encéfalo procedentes de ratones que desarrollan una EET de forma espontánea.
- Objetivos específicos:
    - Determinar mediante la técnica inmunohistoquímica la acumulación de la proteína PDI, un marcador de estrés del retículo endoplásmico, así como la acumulación de Ub^{G76V}-GFP, un marcador del bloqueo de la degradación proteasómica, en encéfalos de ratones $TgU1^+/TgVole^+$

(desarrollan una EET espontánea) y $TgU1^{+}$/$TgVole^{-}$ (no desarrollan una EET espontánea) de distintas edades.

- Comparar los resultados obtenidos entre animales de la misma edad y distinto genotipo y entre animales de distinta edad y mismo genotipo con el fin de determinar si existen diferencias en la acumulación de alguno de los marcadores.
- Evaluar la co-localización de agregados de UbG^{76V}-GFP y ciertas poblaciones celulares mediante la técnica de inmunofluorescencia.

Estudio Nº5: Resistance-associated deer prion protein polymorphisms limit the peripheral PrP^{CWD} deposition (*preliminary manuscript for main article*).

- Objetivo general: Determinar si los alelos H95 y S96 del gen *Prnp* del ciervo de cola blanca (*Odocoileus virginianus*) pueden ejercer un efecto en el fenotipo neuropatológico y/o en la distribución periférica de la PrP^{CWD} en animales inoculados por vía oral con la cepa de ECC Wisc-1.
- Objetivos específicos:
  - Evaluar el perfil de depósito de la PrP^{CWD} en el SNC y en los tejidos periféricos de ciervos de cola blanca de distintos genotipos para el gen *Prnp*: Q95G96/Q95G96 (wt/wt), S96/wt, H95/wt y H95/S96, mediante la técnica de inmunohistoquímica.
  - Comparar el patrón de depósito observado entre los distintos genotipos.

REVISIÓN BIBLIOGRÁFICA

INTRODUCCIÓN

Las enfermedades priónicas o encefalopatías espongiformes transmisibles (EET), tales como el scrapie en ovejas y cabras, la enfermedad crónica caquectizante (ECC) en cérvidos, la encefalopatía espongiforme bovina (EEB) y las que afectan a la especie humana, como son la enfermedad de Creutzfeldt-Jakob (ECJ) o el Kuru, a modo de ejemplo, son procesos neurodegenerativos poco frecuentes, transmisibles, de largo periodo de incubación y que presentan un curso invariablemente fatal. Las EET están producidas por la conversión post-traduccional de la proteína prion celular (PrP^{C}), una glicoproteína que en los mamíferos está codificada de forma fisiológica por el gen *PRNP*, en una isoforma anómala, denominada PrP^{Sc}. Este cambio conformacional le otorga a la PrP^{Sc} una gran resistencia a los procesos de esterilización físicos y químicos, tendencia a la agregación, insolubilidad en detergentes no iónicos y resistencia parcial a la digestión con proteasa (Prusiner, 1982, 1998b).

Las enfermedades priónicas pueden clasificarse en tres grupos en función de su origen:

- Esporádicas o espontáneas: Aquellas que aparecen sin una causa aparente conocida. Sin embargo, en la especie humana son, con diferencia, las más frecuentes. De hecho representan aproximadamente un 85% del total de casos diagnosticados en el ser humano (Ladogana *et al.*, 2005).
- Genéticas o familiares: Asociadas a ciertas mutaciones concretas del gen *PRNP* que resultan en formas anormales de la proteína prion, las cuales tienen una mayor predisposición a adoptar cambios conformacionales aberrantes.
- Adquiridas: Aquellas que aparecen como resultado de la exposición del individuo a los priones, produciéndose, por tanto, una infección con el agente causal.

Independientemente de su origen, las EET presentan una serie de características comunes. Los principales hallazgos neuropatológicos son la gliosis, la pérdida neuronal, el depósito de proteína prion y la degeneración espongiforme del sistema nervioso central (SNC), con la aparición de vacuolas intraneuronales (vacuolización) y en el neuropilo (espongiosis) (Bell and Ironside, 1993; Fraser, 1993). Estos cambios neuropatológicos se presentan con especial frecuencia en determinadas áreas y núcleos nerviosos. En las

distintas especies afectadas por las EET han sido identificadas diferentes cepas y fenotipos del agente etiológico.

De entre todas estas enfermedades es el scrapie, que afecta a ovinos y caprinos, la enfermedad prototipo de las EET, ya que es la enfermedad priónica que se conoce desde hace más tiempo. Sin embargo, las EET han sido descritas en muchas otras especies de mamíferos, especialmente a partir de la crisis de la EEB en los años 80 (Wells *et al.*, 1987), la cual evidenció que el rango de especies susceptibles de desarrollar una EET de forma natural era considerablemente mayor de lo que se creía. Pero lo más relevante fue la aparición de la nueva variante de Creutzfeldt–Jakob en la especie humana (vECJ) que se asoció al consumo de tejidos procedentes de bovinos infectados con EEB (Will *et al.*, 1996; Bruce *et al.*, 1997), lo cual dio lugar a numerosos estudios sobre las enfermedades priónicas y su transmisibilidad. Asimismo, se intensificó la investigación relacionada con los conceptos de cepas priónicas y barrera de transmisión frente a los priones, pues se hizo patente la necesidad de conocer los mecanismos que regulan la transmisibilidad de estas enfermedades entre especies.

Existen muchos aspectos desconocidos de la patogenia de las EET, sin embargo se ha demostrado que la presencia de la PrP^{C} en el SNC es fundamental, no sólo para la generación de la PrP^{Sc}, sino también para que el huésped experimente la neurotoxicidad asociada a los priones (Brandner *et al.*, 1996). Hasta la fecha no han podido detectarse anticuerpos frente al agente causal en los individuos afectados, no existiendo tampoco tratamientos específicos ni vacunas para combatir o prevenir estos procesos. Actualmente, los principales métodos diagnósticos se basan en el estudio *post mortem* del tejido nervioso.

ETIOLOGÍA

El scrapie se describió por primera vez en 1732, sin embargo, fue a partir de 1936 cuando la enfermedad consiguió transmitirse experimentalmente, y en consecuencia se comenzó a especular sobre cuál podría ser la etiología de la enfermedad. A partir de este año se llevaron a cabo diversos estudios con el fin de conocer la verdadera naturaleza del agente infeccioso, y el scrapie se convirtió en el prototipo de las EET. Inicialmente las EET se incluyeron en el grupo de las enfermedades lentivirales, debido a sus largos periodos de incubación y a que el agente causal podía filtrarse a través de filtros

específicos para agentes víricos (Wilson *et al.*, 1950). Sin embargo, posteriormente se comprobó que el agente etiológico de las EET presentaba características únicas, pues era resistente a la mayor parte de tratamientos físicos y químicos capaces de inactivar lentivirus, y además no producía una respuesta inmune en el hospedador. A partir de entonces comenzó a especularse que podría tratarse de un agente que tenía la capacidad de replicarse sin la presencia de ácidos nucleicos (Alper *et al.*, 1967).

En 1967, quince años antes de que la PrP^{C} y la PrP^{Sc} fueran identificadas, se formuló por primera vez la teoría de la "proteína única", sobre la base de la hipótesis de que el agente etiológico de las EET poseía naturaleza proteica y que era capaz de reproducirse sin necesidad de material genético (Griffith, 1967). Basándose en esta teoría, Stanley B. Prusiner introdujo en 1982 el término prion (*proteinaceous infectious particle*), para diferenciar a este nuevo agente infeccioso de otros agentes patógenos, siendo definido como una pequeña partícula proteica infecciosa que no poseía ácidos nucleicos (Prusiner, 1982). Esta partícula proteica, a diferencia de otras proteínas de peso molecular similar, era resistente a la digestión con proteinasa K, por lo que se la denominó PrP^{res} o PrP 27-30, ya que tras la digestión con esta enzima posee un tamaño de 27-30 kD (Bolton *et al.*, 1982). El gen responsable de codificar esta proteína, el gen *PRNP*, fue identificado posteriormente en el genoma del hospedador, descubriéndose, asimismo, que dicha partícula era una fracción procedente de una molécula mayor, que se denominó PrP^{Sc} (Oesch *et al.*, 1985). Así pues la PrP^{Sc} se estableció como el agente causal de las EET considerándose una isoforma anómala de una glicoproteína de membrana codificada de forma fisiológica por el hospedador, la proteína prion celular o PrP^{C}, la cual se expresa fundamentalmente en el tejido nervioso (Chesebro *et al.*, 1985). La PrP^{C}, de forma espontánea o por contacto con la PrP^{Sc}, sufre una modificación post-traduccional, lo cual produce un cambio de conformación que le confiere resistencia a los procesos degradativos, acumulándose en los tejidos del huésped (Prusiner, 1991; Pan *et al.*, 1993).

Sin embargo, esta teoría contradecía el principio de que todo agente infeccioso precisa de material genético para poder replicarse, y además no explicaba de forma convincente la existencia de cepas priónicas. Por estas razones se formuló la teoría del virino, en la cual se propone que el agente causal podría ser una molécula quimérica constituida por una proteína codificada por el hospedador y un pequeño ácido nucleico no codificante propio del agente infeccioso. La teoría del virino fue originalmente formulada por Dickinson y Outram (Dickinson and Outram, 1979) y posteriormente

desarrollada por Kimberlin (Kimberlin, 1982). Esta teoría surgió para intentar explicar la variabilidad que presentaban los distintos aislados de scrapie al ser transmitidos a roedores, pues se consideró que la presencia de ácidos nucleicos en el agente causal podría explicar la aparición de mutaciones, y por tanto la variación fenotípica observada en los animales. Por otro lado, también se postuló que el agente causal de las EET podría ser ADN mitocondrial envuelto por una proteína cuya función sería proteger este material genético (Aiken *et al.*, 1989; Manuelidis, 2007). No obstante, los resultados obtenidos en multitud de estudios posteriores respaldan la teoría de la proteína única, siendo la única teoría aceptada en la actualidad demostrándose también que los priones generados *in vitro,* e incluso los priones recombinantes generados en bacterias son capaces de producir una EET por sí solos (Legname *et al.*, 2004; Castilla *et al.*, 2005; Colby *et al.*, 2009) o en presencia de ciertos lípidos o moléculas de ARN (Wang *et al.*, 2010).

La proteína prion celular: PrP^C

La PrP^C es una glicoproteína de membrana codificada por el gen *PRNP*, el cual se localiza en el cromosoma 20 en el ser humano, en el cromosoma 2 en el caso del ratón y en el cromosoma 13 en los rumiantes (Sparkes *et al.*, 1986). Este gen se ha identificado en numerosas especies y posee una secuencia genética muy conservada entre los vertebrados. El gen *PRNP* forma parte de una familia de genes entre los que también se encuentran el gen *SPRN*, que codifica una proteína denominada shadoo (Sho), y el gen *PRND*, que codifica la proteína doppel (Dpl). Estas dos proteínas comparten muchas características estructurales con la PrP^C, y se considera que su estudio podría resolver numerosas incógnitas sobre la biología de los priones (Watts *et al.*, 2007).

En la mayor parte de las especies el gen *PRNP* está formado por dos exones separados por un intrón de 10 kb, aunque el exón 1 no se traduce. Sin embargo, en ratones, ovejas y ratas este gen posee tres exones (Westaway *et al.*, 1994a). Al inicio de la biosíntesis de la PrP^C el gen *PRNP* se transcribe en el núcleo y el mRNA correspondiente comienza a traducirse en los ribosomas hasta alcanzar un péptido señal de 22 aminoácidos. Este péptido señal se traslada al interior del retículo endoplásmico rugoso (RER) donde se sintetiza el resto de una proteína de 253 aminoácidos (254 en algunas especies) (Cohen, 1999; Harris, 2003). En el RER se produce la formación de un puente disulfuro entre los residuos de cisteína 179 y 214 y al mismo tiempo puede producirse la adición de N-glicanos a los residuos aminoacídicos 181 y/o 197, lo cual dará lugar a las

tres glicoformas en las que puede presentarse esta proteína: la forma no glicosilada, la monoglicosilada y la diglicosilada (Caughey *et al.*, 1989; Monari *et al.*, 1994). Cuando se completa la síntesis, se elimina el péptido señal de 22 aminoácidos del extremo aminoterminal de la PrP^C y se añade el anclaje glicosilfosfatidilinositol (GPI) al extremo carboxiloterminal (Stahl *et al.*, 1987; Haraguchi *et al.*, 1989). En el tránsito a través del aparato de Golgi se produce la incorporación de residuos acídicos (Mays and Soto, 2016) y posteriormente la PrP^C madura se traslada a la membrana celular, localizándose generalmente en las balsas lipídicas (Taraboulos *et al.*, 1992; Vey *et al.*, 1996). Una vez allí la PrP^C transita entre la superficie y el interior celular por medio de endosomas (Vey *et al.*, 1996; Peters *et al.*, 2003; Mays and Soto, 2016).

Los estudios estructurales y bioquímicos muestran que la PrP^C consta de una serie de regiones bien diferenciadas:

- Una zona no estructurada en el extremo aminoterminal (N-terminal) caracterizada por la presencia de una serie de repeticiones de un octapéptido (PHGGGWGQ).
- Un dominio globular estructurado en el extremo carboxiloterminal (C-terminal), compuesto por tres hélices α y dos láminas β antiparalelas de cuatro aminoácidos cada una. En esta zona existe un puente disulfuro entre dos residuos de cisteína (C179 y C214) localizados en las α-hélices 2 y 3. Asimismo, están presentes dos sitios de N-glicosilación, en los residuos N181 y N197 (posiciones correspondientes a la PrP^C humana) y el anclaje GPI, localizado en el aminoácido S231 en casi todas las especies de mamíferos.
- Una región hidrofóbica (entre los residuos 112-145) que conecta los dominios N- y C- terminal y que se encuentra altamente conservada entre las distintas especies.

La expresión de la PrP^C en los tejidos es necesaria para el desarrollo de las EET. Por ello, los ratones *knock-out* para el gen *PRNP* son resistentes a la inoculación con priones (Sailer *et al.*, 1994). Los niveles de expresión de la PrP^C en el encéfalo poseen también influencia en el periodo de incubación de las enfermedades priónicas, siendo el nivel de expresión de la PrP^C inversamente proporcional a la duración del periodo de incubación (Prusiner *et al.*, 1990; Bueler *et al.*, 1994). La PrP^C se expresa

fundamentalmente en el SNC, aunque también se encuentra presente en muchos otros tipos de células y tejidos, aunque en niveles más bajos. En el SNC la PrP^C está presente fundamentalmente en las neuronas y en las células de la glía (Moser *et al.*, 1995; Weissmann *et al.*, 1998; Adle-Biassette *et al.*, 2006; Lima *et al.*, 2007), aunque también se expresa en otros tipos de células, como las células endoteliales de los vasos sanguíneos cerebrales (Adle-Biassette *et al.*, 2006). Asimismo, sus niveles de expresión también son elevados en el sistema nervioso periférico (SNP), estando presente en los ganglios nerviosos de la médula espinal (Tremblay *et al.*, 2007; Peralta *et al.*, 2012; Ganley *et al.*, 2015), en los axones sensitivos y motores (Manson *et al.*, 1992) y en las células de Schwann (Follet *et al.*, 2002). Fuera del sistema nervioso la PrP^C se expresa en el sistema linforreticular (SLR), fundamentalmente en linfocitos y células dendríticas foliculares (Aguzzi and Heikenwalder, 2006) y ha sido detectada en muchos otros órganos, como el corazón, el páncreas, el intestino, el hígado, los riñones, el pulmón y el útero (Moudjou *et al.*, 2001; Peralta and Eyestone, 2009; Garza *et al.*, 2014; Hedman *et al.*, 2016).

Si bien la estructura y localización celular de la PrP^C se han descrito ampliamente, sus funciones fisiológicas siguen sin conocerse con exactitud. Una buena parte de los estudios realizados para conocer las funciones de la PrP^C se han encaminado a averiguar las supuestas propiedades protectoras de esta proteína frente al estrés celular. Inicialmente se observó que la PrP^C protegía a las células cuando en cultivos celulares eran privadas del suero presente en el medio de cultivo, lo cual activa la apoptosis dependiente de la vía mitocondrial a través de una proteína denominada Bax (Kuwahara *et al.*, 1999; Kim *et al.*, 2004; Wu *et al.*, 2008). También, se observó que la PrP^C inhibía específicamente a la proteína Bax, protegiendo así a los tejidos frente a la neurodegeneración (Roucou *et al.*, 2004). Además de sus efectos directos sobre la apoptosis, se ha sugerido que la PrP^C también protege a las células frente al estrés oxidativo. Así pues, la expresión de PrP^C en cultivos de neuronas, astrocitos y otras líneas celulares se ha asociado con menores niveles de daño tras la exposición a diversas toxinas oxidativas (Brown *et al.*, 1997; Anantharam *et al.*, 2008; Dupiereux *et al.*, 2008). En lo que se refiere a su efecto protector frente al estrés celular, la PrP^C ha sido relacionada con la respuesta frente al estrés del retículo endoplásmico (RE), que se produce por la acumulación de proteínas desplegadas o malplegadas dentro de este orgánulo. Aunque este punto se desarrollará más adelante, las células responden al estrés del RE desencadenando una respuesta que consiste en un aumento de la expresión de chaperonas, una activación de la vía de degradación de

proteínas asociadas al RE y una inhibición global de la síntesis de proteínas (Castle and Gill, 2017). Se ha observado que la eliminación de la expresión de la PrP^{C} en varias líneas celulares cancerígenas da como resultado un aumento de la muerte celular en respuesta a toxinas que causan estrés del RE (Dery *et al.*, 2013).

La PrP^{C} también ha sido ampliamente relacionada con la diferenciación celular. Se considera que esta proteína podría cumplir importantes funciones en los procesos de neurogénesis, neuritogénesis, adhesión celular, expresión de proteínas del citoesqueleto, desarrollo axonal y mantenimiento de la sustancia blanca (Aguzzi *et al.*, 2008; Castle and Gill, 2017). Aunque menos estudiadas, se han descrito muchas otras funciones que podrían estar asociadas a la PrP^{C}. Entre ellas destacan su posible papel en la excitabilidad neuronal mediante su interacción con neurotransmisores, la regulación del ritmo circadiano, la modulación de la homeostasis del cobre, la participación en la respuesta inmune regulando las funciones de los mastocitos y los linfocitos T, el mantenimiento de la homeostasis mitocondrial y la regulación de los niveles de β-amiloide y tau (Castle and Gill, 2017).

La proteína prion patológica: PrP^{Sc}

La PrP^{C} y la PrP^{Sc} comparten la misma secuencia de aminoácidos, sin embargo, difieren en su conformación. Si bien la estructura tridimensional de la PrP^{C} se conoce desde hace años (Riek *et al.*, 1996), la de la PrP^{Sc} sigue siendo en parte desconocida y se considera un factor muy importante a aclarar, ya que ayudaría a comprender los mecanismos moleculares implicados en la propagación de los priones. Los principales problemas al respecto son la insolubilidad y la predisposición de la PrP^{Sc} a formar agregados, lo que impide la utilización de la mayor parte de las técnicas de alta definición destinadas a conocer la estructura tridimensional de las proteínas, como son la espectroscopía mediante resonancia magnética nuclear (RMN) y la cristalografía de rayos X. Así pues, una gran parte de los datos sobre la estructura de la PrP^{Sc} proceden de técnicas de baja resolución y de modelos moleculares predictivos, los cuales han sugerido estructuras tridimensionales significativamente distintas (Requena and Wille, 2014). El modelo estructural de la PrP^{Sc} que más se ha discutido en la literatura científica es aquel que propone que durante el proceso de conversión de la PrP^{C} en PrP^{Sc}, la mayor parte de los cambios se producen en la región N-terminal de la proteína, la cual adopta la conformación de lámina plegada β, mientras que la región C-terminal mantiene la

conformación de hélice α de la PrP^{C} (Pan *et al.*, 1993; Peretz *et al.*, 1997). Así pues, se ha aceptado que la estructura de la PrP^{Sc} estaba formada por aproximadamente un 30% de hélices α y un 40% de láminas plegadas β (Pan *et al.*, 1993; Zhang *et al.*, 1995). Sin embargo, los estudios más recientes sostienen que no hay evidencia de que existan hélices α en la PrP^{Sc} (Smirnovas *et al.*, 2011). De hecho, dos de los modelos más recientes descritos: el modelo PIRIBS (*Parallel In Register Intermolecular β-Sheet*), basado en el estudio de PrPs recombinantes mediante RMN (Groveman *et al.*, 2014) y el modelo de β-solenoide de cuatro escalones superpuestos, que describe la estructura de la PrP^{res} a través del uso de criomicroscopía electrónica (Vazquez-Fernandez *et al.*, 2016) coinciden en señalar que la proteína prion patológica está enteramente compuesta por láminas β.

Como se ha comentado, la conversión de la PrP^{C} en PrP^{Sc} es un proceso post-traduccional que no sólo modifica la estructura de la proteína, sino que también afecta profundamente a las propiedades bioquímicas y biofísicas de esta. El malplegamiento de la PrP^{Sc} le confiere una gran tendencia a la agregación, resistencia parcial a la digestión con proteinasa K, la hace insoluble en detergentes y le proporciona una gran resistencia al calor, a las radiaciones ionizantes y ultravioletas y a la desinfección con la mayor parte de agentes químicos (Prusiner, 1998b; Caughey and Chesebro, 2001). De hecho, como se ha mencionado, la digestión de la PrP^{Sc} con proteinasa K da lugar a un fragmento C-terminal denominado PrP^{res} o PrP 27-30, que es el marcador bioquímico más utilizado para la detección y caracterización de las distintas cepas priónicas (Gielbert *et al.*, 2009).

Conversión de la PrP^{C} en PrP^{Sc}

Las enfermedades priónicas, como se ha descrito, pueden tener un origen espontáneo, familiar o bien ser adquiridas como resultado de una infección por priones. Pero independientemente de su origen, la propagación de los priones en un individuo depende de la conversión de la PrP^{C} en la isoforma patógena PrP^{Sc}. Los mecanismos implicados en esta conversión no han sido totalmente esclarecidos y se han propuesto dos modelos distintos para intentar explicar el proceso (Figura 1):

-Modelo de plegamiento asistido por molde: Este modelo fue originalmente propuesto por Prusiner y considera que el paso crítico en el proceso de conversión es la formación de un heterodímero entre la PrP^{C} y la PrP^{Sc}, actuando esta última como un molde que induce la transformación de la PrP^{C}. Estas nuevas moléculas de PrP^{Sc} provocarían un fenómeno de retroalimentación, replicándose mientras exista PrP^{C} en el medio que actúe

como sustrato. Finalmente, las moléculas de PrP^{Sc} se agregarían y formarían fibrillas de amiloide, las cuales no son esenciales para la replicación de los priones (Prusiner, 1991).
-Modelo de nucleación sembrada o modelo de nucleación-polimerización: Esta hipótesis establece que la PrP^{C} y la PrP^{Sc} se encuentran en un estado de equilibrio termodinámico que en condiciones fisiológicas está desplazado hacia la PrP^{C}. Según este modelo, varias moléculas de PrP^{Sc} se unirían para formar un núcleo o semilla al cual se irían uniendo progresivamente nuevas moléculas de PrP^{Sc}, formándose unos agregados de tipo amiloide. La fragmentación de estos agregados aumentaría el número de núcleos o semillas que, a su vez, pueden reclutar nuevas moléculas de PrP^{Sc} y producir un cambio masivo sobre las moléculas de PrP^{C} , resultando en la replicación del prión y confiriéndole su capacidad infecciosa (Jarrett and Lansbury, 1993; Glatzel and Aguzzi, 2001). Existen evidencias experimentales que apoyan esta hipótesis, ya que se ha demostrado que la PrP^{C} puede convertirse en PrP^{Sc} cuando es incubada con PrP^{res} procedente de animales infectados (Kocisko *et al.*, 1994; Horiuchi and Caughey, 1999; Caughey, 2001). Asimismo, la técnica de la amplificación cíclica de proteínas mal plegadas (*protein misfolding cyclic amplification*, PMCA), que fue desarrollada posteriormente, se basa en este modelo. Esta técnica, a través de rondas sucesivas de sonicación e incubación, es capaz de amplificar la PrP^{Sc} de forma indefinida cuando un homogeneizado de encéfalo no infectado, que actuaría como sustrato, se incuba con pequeñas cantidades de homogeneizado procedente de un animal infectado (Castilla *et al.*, 2005).

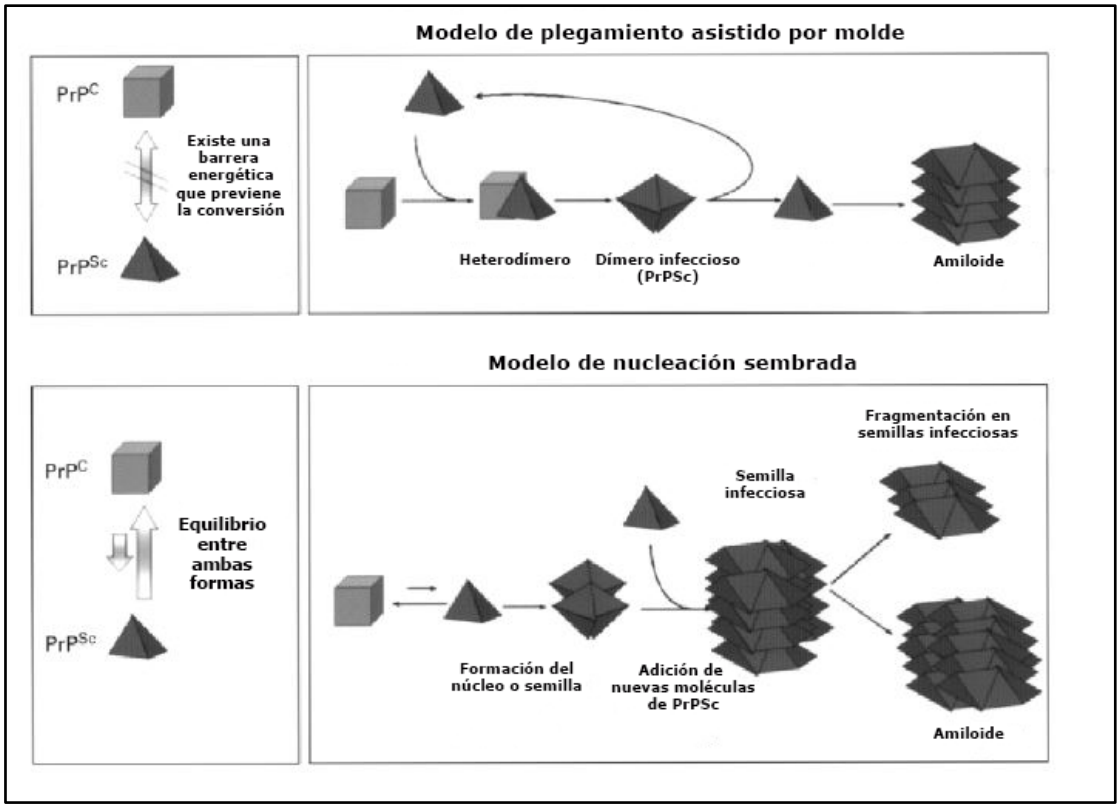

Figura 1: Modelos propuestos para explicar la conversión conformacional de la PrP^{C} en PrP^{Sc}. Adaptado de Aguzzi and Heppner, 2000.

ENFERMEDADES PRIÓNICAS: SUSCEPTIBILIDAD Y RESISTENCIA

La aparición de la teoría de la proteína única supuso un avance muy significativo en la investigación de las EET. Sin embargo, hizo más difícil explicar el concepto de la existencia de cepas y los mecanismos implicados en la transmisibilidad entre especies. La primera enfermedad priónica de la que se tiene constancia es el scrapie de ovejas y cabras. Posteriormente, a medida que nuevas EET se iban descubriendo, la lista de especies susceptibles a estas enfermedades fue incrementándose. Con el fin de comprender la patogenia y la transmisibilidad de las enfermedades priónicas se llevaron a cabo numerosas infecciones experimentales (Chianini *et al.*, 2013). Estos experimentos comenzaron a realizarse incluso antes de que se propusiera que las EET estaban producidas por proteínas infecciosas, y ya desde entonces se comprobó que existían ciertas especies que no desarrollaban clínica tras la inoculación experimental del agente causal. Así, por ejemplo, en 1976 se observó que el conejo era resistente a la inoculación con el agente del scrapie (Barlow and Rennie, 1976). Gibbs y Gadjusek, por su parte,

describieron la distinta susceptibilidad que presentaban las diferentes especies a lo que ellos denominaban encefalopatías espongiformes víricas subagudas (Gibbs and Gajdusek, 1973).

La aparición de la EEB (Wells *et al.*, 1987) fue un hecho clave que contribuyó enormemente no solo a descubrir el potencial zoonótico de estos agentes (Bruce *et al.*, 1997) sino también a conocer mejor el rango de especies susceptibles a las enfermedades priónicas. Muchas especies en las que no se habían descrito previamente casos de EET se vieron afectadas tras la epidemia de la EEB. Asimismo, se comprobó la resistencia de otras especies a la infección natural por priones ya que, por ejemplo, si bien se detectó EEB en el ganado caprino (Eloit *et al.*, 2005; Spiropoulos *et al.*, 2011), no se describieron casos en cerdos, a pesar de que estuvieron igualmente expuestos al agente infeccioso (Chianini *et al.*, 2013).

Por ello, se considera de gran importancia conocer los mecanismos implicados en la susceptibilidad o resistencia de las distintas especies a las enfermedades priónicas. Si bien en muchas ocasiones la susceptibilidad de una especie a las EET depende en gran medida de la cepa priónica transmitida, otros factores como el genotipo del huésped también juegan un papel fundamental. Es por ello que se ha investigado el papel de ciertas mutaciones puntuales en la secuencia del gen *PRNP* en la resistencia a las EET, llegándose a proponer algunas de ellas como posibles candidatas para el desarrollo de terapias génicas frente a estas enfermedades (Perrier *et al.*, 2002; Otero *et al.*, 2017).

Enfermedades priónicas animales: Hospedadores naturales

De entre todas las enfermedades priónicas, el scrapie, tembladera o enfermedad de prurigo lumbar del ganado ovino y caprino es la que se conoce desde hace más tiempo, ya que se describió por primera vez en Inglaterra en 1732. La primera descripción de la enfermedad en Alemania se realizó en1750, y ya se consideraba su posible naturaleza infecciosa (Leopoldt, 1750). En el año 1936 se demostró por primera vez la transmisibilidad del scrapie mediante la inoculación experimental de ovejas y cabras con tejido nervioso de ovejas infectadas, comprobándose el largo periodo de incubación de esta enfermedad ya que en algunos animales el scrapie no se desarrolló hasta 2 años tras la inoculación (Cuille and Chelle, 1936, 1938a, 1938b). Desde entonces ha sido la enfermedad priónica más estudiada y la que está más ampliamente distribuida en todo el mundo.

En el año 1998, la forma atípica del scrapie, que se denominó Nor98, fue observada por primera vez en Noruega en el ganado ovino (Benestad *et al.*, 2003). Sin embargo, estudios retrospectivos revelaron que ya se habían producido casos de scrapie atípico en el Reino Unido a finales de los 80 (Bruce *et al.*, 2007). Se diferencia del scrapie clásico clínica y epidemiológicamente, y presenta sus propias características bioquímicas e histopatológicas, estando distribuido también mundialmente y presentando una incidencia similar a la del scrapie clásico (Fast and Groschup, 2013).

En 1947, en Estados Unidos, se detectó una rara enfermedad neurológica que afectaba a visones criados en granjas. Años más tarde este proceso se identificó como una enfermedad priónica, la encefalopatía transmisible del visón (ETV) (Barlow, 1972). Posteriormente la enfermedad se localizó en diversas zonas de América y Europa (Marsh and Hadlow, 1992). Sin embargo, los brotes de ETV son muy poco frecuentes, ocurriendo el último de ellos en 1985. Se ha especulado que el origen de la ETV en esta especie podría estar relacionado con la ingestión de carne contaminada con priones, probablemente de ovejas infectadas con scrapie (Marsh and Bessen, 1993). No obstante, el origen de la ETV sigue siendo desconocido.

En el año 1967 se identificó por primera vez, en un ciervo mula cautivo en Colorado, una enfermedad neurodegenerativa que fue formalmente diagnosticada como una EET en 1978: la ECC (Williams and Young, 1980). Desde entonces la ECC se diseminó a distintas especies de cérvidos de Estados Unidos y Canadá, alcanzando prevalencias muy altas en algunos estados (Williams, 2005). Esta enfermedad también se ha detectado en Corea del Sur y, en el año 2016, ha sido descrita por primera vez en Europa (Benestad *et al.*, 2016), por lo que actualmente se considera una enfermedad emergente en el continente. El origen de la ECC se desconoce a día de hoy, pero el hecho de que sea la única enfermedad priónica que afecta tanto a animales domésticos como silvestres, unido a su gran capacidad de diseminación, han hecho que el ciclo de transmisión de la ECC entre especies, la persistencia del agente en el ambiente y los factores que determinan la emergencia de nuevas cepas despierten un gran interés en la investigación de esta enfermedad (Miller and Williams, 2003; Duque Velasquez *et al.*, 2015).

Sin embargo, las enfermedades priónicas y su posible transmisión entre especies se convirtieron en motivo de preocupación pública tras la aparición de la EEB. En los

años 80 se descubrió esta enfermedad en el ganado bovino del Reino Unido (Wells *et al.*, 1987), relacionándose la aparición de la enfermedad con la alimentación del vacuno con harinas de carne y hueso procedentes de ovejas infectadas con scrapie (Wilesmith *et al.*, 1988; Wilesmith *et al.*, 1991). La epidemia se propagó rápidamente, afectando a miles de animales y extendiéndose a otros países (Brown *et al.*, 2001). No obstante, la enorme repercusión sanitaria, económica y política de la EEB fue debida a la aparición de la variante de la enfermedad de Creutzfeldt-Jakob (vECJ) en la especie humana en los años 90 y su asociación con el consumo de productos de origen bovino contaminados con priones de EEB (Will *et al.*, 1996; Bruce *et al.*, 1997). Tras las medidas instauradas por la Comisión Europea con el fin de prevenir, controlar y erradicar la EEB se consiguió una drástica disminución de la prevalencia de la enfermedad. Sin embargo, la intensa vigilancia activa de la EEB en Europa y la mejora en los métodos de diagnóstico condujeron a la identificación, en la década del 2000, de dos nuevas formas de EEB en el ganado bovino, que se denominaron formas atípicas de la EEB (Ducrot *et al.*, 2008). La EEB de tipo L o EEB-L se detectó por primera vez en Italia en dos animales (Casalone *et al.*, 2004). Estos bovinos presentaban diferencias significativas en la distribución de las lesiones encefálicas con respecto a los animales infectados con EEB clásica (EEB-C). Por otro lado, la EEB de tipo H (EEB-H), fue descrita por primera vez en Francia (Biacabe *et al.*, 2004). Actualmente siguen declarándose casos de EEB atípica en diversos países europeos (Organización Mundial de Sanidad Animal, 2016). Estos casos se diagnostican en el ganado bovino adulto y se desconoce su origen, aunque se ha propuesto que podrían tratarse de EET que aparecen de forma esporádica o espontánea en estos animales (Seuberlich *et al.*, 2010).

Tras la epidemia de EEB numerosas especies desarrollaron una enfermedad priónica de forma natural. Varios animales pertenecientes a siete especies distintas de ungulados exóticos (niala, órice del Cabo, gran kudú, órice blanco, órice árabe, eland común y bisonte) padecieron una enfermedad priónica similar a la EEB (Jeffrey and Wells, 1988; Wilesmith *et al.*, 1988; Fleetwood and Furley, 1990; Kirkwood *et al.*, 1990; Kirkwood and Cunningham, 1994; Sigurdson and Miller, 2003). De forma paralela, la enfermedad se descubrió también en primates no humanos (Bons *et al.*, 1999). Por otro lado, la encefalopatía espongiforme felina (EEF) se observó por primera vez en gatos en 1990 (Leggett *et al.*, 1990) y desde 1992 hasta 2005 esta enfermedad fue diagnosticada en seis especies de felinos más (guepardo, león, ocelote, puma, tigre y leopardo)

(Kirkwood and Cunningham, 1994; Williams *et al.*, 2001). Estudios epidemiológicos demostraron que todas estas especies de rumiantes, primates y felinos habían consumido piensos que contenían harinas de carne y hueso de rumiantes, carne de vacuno o bien habían estado próximos a bovinos infectados con EEB (Kirkwood *et al.*, 1993; Kirkwood and Cunningham, 1994; Sigurdson and Miller, 2003). Además, se han detectado casos naturales de EEB en pequeños rumiantes, concretamente en dos cabras. Uno de los casos se diagnosticó en una cabra doméstica en Francia en el año 2005 (Eloit *et al.*, 2005) y, en 2011, se confirmó otro caso en el Reino Unido, en un caprino que había sido diagnosticado de scrapie en 1990 e identificado de forma retrospectiva como sospechoso de EEB (Spiropoulos *et al.*, 2011).

Finalmente, en el año 2018, se ha detectado en Algeria una enfermedad priónica que afecta a los dromedarios (*Camelus dromedarius*). Se considera que un 3.1% de los dromedarios que se sacrificaron en el matadero de Ouargla entre 2015 y 2016 presentaban signos clínicos compatibles con esta enfermedad priónica. El diagnóstico fue confirmado tras la observación de degeneración espongiforme y depósito de PrP^{Sc} en el SNC de animales afectados. Además, se ha demostrado que los priones causantes de esta enfermedad presentan características bioquímicas distintas a la EEB y el scrapie (Babelhadj *et al.*, 2018).

Enfermedades priónicas humanas

El ser humano también es un hospedador natural de las EET. Actualmente se han descrito nueve enfermedades priónicas humanas distintas las cuales, como se ha mencionado, pueden tener origen esporádico o espontáneo, genético o familiar o ser adquiridas como resultado de una infección. Las EET humanas esporádicas incluyen la forma espontánea de la enfermedad de Creutzfeldt-Jakob (eECJ), el insomnio esporádico fatal (IEF) y la prionopatía variable sensible a la proteasa (PVSPr) (Imran and Mahmood, 2011). Las enfermedades priónicas genéticas o familiares, que están producidas por mutaciones autosómicas dominantes del gen *PRNP*, incluyen la enfermedad de Creutzfeldt-Jakob familiar (fECJ), el insomnio familiar fatal (IFF) y la enfermedad de Gerstmann-Sträussler-Scheinker (GSS). Por último, las EET humanas adquiridas, y que representan únicamente el 5% de las enfermedades priónicas diagnosticadas en el ser humano, son el kuru, asociado al canibalismo ritual; la enfermedad de Creutzfeldt-Jakob iatrogénica, resultado de la exposición accidental a priones durante procedimientos

médicos y quirúrgicos, y la ya mencionada vECJ, asociada al consumo de productos contaminados con EEB (Imran and Mahmood, 2011).

Sin embargo, de entre todas las enfermedades priónicas humanas la más frecuente es la eECJ, representando aproximadamente un 85% de los casos de EET diagnosticados en el ser humano (Masters *et al.*, 1979; Safar, 2012). Identificada por primera vez en los años 20 (Creutzfeldt, 1920; Jakob, 1921), el origen de esta enfermedad sigue siendo desconocido. No obstante, los datos epidemiológicos sugieren que no se debe a ninguna causa infecciosa y se considera que se produce por una conversión aleatoria de la PrP^{C} en la forma patógena, la cual se acumula produciendo la neurodegeneración (Will and Ironside, 2017).

Transmisión experimental de las enfermedades priónicas

A lo largo de los años se han utilizado muchas especies como modelo experimental de la EET. Estos estudios han servido no sólo para demostrar la susceptibilidad a las EET de un amplio rango de hospedadores en los que no se habían observado casos naturales, sino también para conocer los aspectos clínicos, neuropatológicos, genéticos y bioquímicos de las enfermedades priónicas. Entre estos modelos experimentales los roedores siempre han sido los más empleados y útiles, por su sensibilidad a las EET y su rapidez en el desarrollo de signos clínicos.

Sin embargo, se ha demostrado la vulnerabilidad a las enfermedades priónicas de muchas otras especies de mamíferos en las que no se han descrito EET naturales.

Mamíferos susceptibles a la transmisión experimental de las EET: Modelos experimentales

Como se ha mencionado, la primera transmisión experimental de una enfermedad priónica se realizó en los años 30, lo que permitió confirmar la naturaleza transmisible del scrapie (Cuille and Chelle, 1936, 1938b). Estos estudios fomentaron que, años más tarde, se demostrara la transmisibilidad de las EET humanas con la inoculación del kuru y la eECJ en primates no humanos (Gajdusek *et al.*, 1966; Gajdusek *et al.*, 1968; Gibbs *et al.*, 1968).

El hámster fue un modelo muy popular desde los años 70 para la realización de muchos tipos de estudios sobre las EET. Sin embargo, fueron reemplazados gradualmente por los ratones transgénicos a partir de los 90 (Brandner and Jaunmuktane, 2017). El

hámster ha sido un modelo experimental fundamental para comprender la patogenia de las enfermedades priónicas, especialmente los mecanismos de diseminación del prión, tanto desde la periferia al SNC como dentro del propio SNC (Kimberlin and Walker, 1986; Jendroska *et al.*, 1991).

Sin embargo, fueron los modelos murinos transgénicos los que permitieron dar un paso de gigante en la investigación de las EET. El primer ratón transgénico diseñado para la investigación de las enfermedades priónicas expresaba transgenes procedentes del hámster. Su creación fue un paso muy importante hacia la comprensión del concepto de la barrera de transmisión, ya que se comprobó la importancia de la secuencia del gen *PRNP* en la misma (Scott *et al.*, 1989). Sin embargo, el mayor avance en este campo se consiguió en 1992 con la creación del ratón *knock-out* para el gen *PRNP* (*Prnp*$^{0/0}$) (Bueler *et al.*, 1992). Estos animales fueron la base para la creación de incontables líneas de ratones transgénicos que expresan numerosos transgenes sin que éstos interfieran con la PrP^{C} del hospedador (Weissmann and Bueler, 2004; Brandner and Jaunmuktane, 2017). Además, el modelo *Prnp*$^{0/0}$ demostró un hecho de gran importancia: que la expresión de la PrP^{C} es necesaria para que los priones se transmitan y propaguen en un individuo (Sailer *et al.*, 1994).

Son muchas las especies en las que se han conseguido reproducir experimentalmente las EET. A modo de ejemplo se pueden citar los hurones (Bartz *et al.*, 1998), mofetas, mapaches (Eckroade *et al.*, 1973) y cerdos, en los que la EEB se transmite eficientemente (Dawson *et al.*, 1990; Hedman *et al.*, 2016).

Sin embargo, uno de los modelos más interesantes para el estudio de las enfermedades priónicas es el *bank vole* o topillo rojo (*Myodes glareolus*). El gen *PRNP* de este roedor silvestre es polimórfico en el codón 109, codificando isoleucina (I) o metionina (M), y se considera que este factor es determinante para la gran susceptibilidad que presenta esta especie a las EET (Cartoni *et al.*, 2005; Watts *et al.*, 2012). El *bank vole* ha sido utilizado en numerosos estudios de transmisión, en los que se ha demostrado que esta especie es vulnerable a la inoculación con cepas de scrapie que no son transmisibles a los modelos murinos convencionales y transgénicos (Di Bari *et al.*, 2008). Asimismo, se ha demostrado que son sensibles a la inoculación con eECJ (Nonno *et al.*, 2006) y, sorprendentemente, a la inoculación con numerosas cepas de GSS, una enfermedad que se consideraba poco o nada transmisible (Pirisinu *et al.*, 2016). Por otro lado, la

adaptación de la ECC al *Myodes glareolus* condujo al aislamiento de la cepa priónica más rápida que se conoce (CWD-vole), que presenta periodos de supervivencia de 35 días aproximadamente (Di Bari *et al.*, 2013). Todos estos factores han contribuido a que se considere a esta especie como "el receptor universal del prión"(Watts *et al.*, 2014). No obstante, la importancia de este roedor radica en su valor como modelo experimental de las enfermedades priónicas espontáneas. En el año 2012 se observó que los ratones transgénicos que sobreexpresan la PrP^{C} del *bank vole* con isoleucina en el codón 109 (BVPrPI109) desarrollan una enfermedad priónica espontánea y transmisible, posiblemente debido a la inherente predisposición al malplegamiento que posee la PrP^{C} del *bank vole* (Watts *et al.*, 2012).

Mamíferos resistentes o poco susceptibles a las enfermedades priónicas

Como se ha descrito, las EET se han transmitido experimentalmente a numerosas especies de mamíferos. Sin embargo, entre los mamíferos existen especies que han sido descritas como resistentes o muy poco susceptibles a las enfermedades priónicas. El concepto de especies resistentes a las EET surgió tras la crisis de la EEB, cuando se observó que ciertos grupos de animales que también habían estado expuestos a alimentos contaminados no habían desarrollado ninguna enfermedad (Fernandez-Borges *et al.*, 2012). Estas especies incluyen a los lepóridos (Vorberg *et al.*, 2003), los équidos (Khan *et al.*, 2010) y los cánidos (Polymenidou *et al.*, 2008).

El conejo ha sido, de entre las supuestas especies resistentes, la más estudiada. Diversos experimentos, en los que se utilizaron tanto cepas priónicas animales como humanas, fracasaron al intentar reproducir una EET en esta especie (Gibbs and Gajdusek, 1973; Barlow and Rennie, 1976). Estas evidencias, junto con posteriores estudios de la estructura de la PrP^{C} del conejo, hicieron que los lepóridos fueran considerados especies resistentes a las enfermedades priónicas durante décadas (Fernandez-Funez *et al.*, 2011; Zhang, 2011). Sin embargo, tras el desarrollo de nuevas técnicas como la PMCA (Castilla *et al.*, 2005) se demostró que los conejos no son completamente resistentes a la infección por priones.

De hecho, se observó que estos animales eran capaces de desarrollar una EET tras ser inoculados con PrP^{Sc} de conejo generada por PMCA y que los cerebros de los animales infectados transmitían la enfermedad al inocularse a otros conejos (Chianini *et al.*, 2012).

No obstante, si bien no pueden considerarse completamente resistentes a las EET, son muy poco susceptibles.

Además, se ha conseguido reproducir una EET en ratones transgénicos que expresan la PrP^{C} del caballo (TgEq). Sin embargo, estos animales, aunque no son totalmente resistentes, presentan una susceptibilidad a las enfermedades priónicas muy particular, mostrando un fenómeno replicativo muy inusual que se ha denominado amplificación de priones no adaptativa (*nonadaptative prion amplification*, NAPA). De este modo se consiguió reproducir una enfermedad priónica en ratones TgEq al ser inoculados con una cepa de scrapie, generándose, por tanto, PrP^{Sc} equina en los encéfalos de estos animales. Sin embargo, la inoculación de esta PrP^{Sc} equina no consiguió transmitir la enfermedad a otros ratones TgEq, pero se transmitió eficientemente a ratones que expresaban la PrP^{C} ovina. Por ello, se propuso el término NAPA para describir a la transmisión interespecie de las EET sin adaptación al nuevo huésped (Bian *et al.*, 2017).

En el año 2010 se realizó un estudio en el que se utilizaron PrP recombinantes procedentes de conejo, caballo y perro. Utilizando una técnica espectroscópica se midió la cantidad de confórmeros de tipo β que se formaban tras someter a estas PrP a distintas condiciones de pH y concentración de urea, lo cual permitía clasificar estas proteínas en función de su predisposición al malplegamiento. Este estudio demostró que la más susceptible era la PrP del conejo, seguida de la del caballo y por último la del perro, que era la menos propensa a adoptar una conformación anómala (Khan *et al.*, 2010). Estos resultados, junto con los obtenidos en otros estudios *in vitro* en los que se ha revelado la enorme dificultad que supone malplegar la PrP^{C} del perro (Vidal *et al.*, 2013) han hecho que los cánidos sean considerados como los mamíferos más resistentes a las enfermedades priónicas.

Otras especies resistentes a las EET: las aves

Las aves domésticas también estuvieron expuestas a la infección por priones durante la epidemia de EEB, pues consumieron piensos que contenían harinas de carne de rumiantes contaminadas. Sin embargo, no se detectaron casos de EET aviares, con la excepción de unos casos sospechosos observados en avestruces procedentes de dos zoos del norte de Alemania. Estos animales desarrollaron un proceso neurodegenerativo con ataxia y pérdida del equilibrio, viéndose cambios espongiformes en el encéfalo tras la necropsia. Sin embargo la etiología no pudo ser establecida con certeza (Schoon *et al.*,

1991). Posteriormente se llevó a cabo un estudio que pretendía comprobar si los pollos domésticos podían o no infectarse con EEB. Para ello inocularon con EEB dos grupos de animales por vía intracerebral e intraperitoneal respectivamente y, con el fin de imitar la exposición oral por consumo de pienso infectado, también inocularon un grupo de animales directamente en el esófago. A pesar de que algunos pollos desarrollaron signos neurológicos con el tiempo, no se observaron depósitos de PrP^{Sc} en el encéfalo, los resultados del Western Blot fueron negativos y, tras inocular encéfalo de estos animales en ratones, tampoco se consiguió reproducir la enfermedad. Por lo tanto concluyeron que no existía evidencia de que los pollos pudieran infectarse por EEB (Moore *et al.*, 2011).

CEPAS PRIÓNICAS

De entre las características únicas que presentan las enfermedades priónicas, una de las más interesantes y controvertidas es sin duda la existencia de diferentes cepas de agentes priónicos. El término "cepa priónica" fue establecido como analogía de otros agentes infecciosos, ya que los individuos afectados por una EET pueden desarrollar distintas patologías desde el punto de vista clínico y bioquímico, propiedades que se mantienen en pases sucesivos en otros individuos. La primera evidencia experimental de la existencia de cepas priónicas se observó en cabras infectadas con scrapie en un estudio realizado por Pattison y Millson en el que describieron la aparición de dos fenotipos clínicos completamente distintos en estos animales, que habían sido infectados con el mismo aislado de scrapie (Pattison and Millson, 1961). Sin embargo, inicialmente, este fenómeno fue utilizado como una de las evidencias más importantes en contra de la teoría de la proteína única ya que el concepto clásico de cepa implica la existencia de material genético en el agente infeccioso (Chesebro, 1998; Soto and Castilla, 2004). No obstante, actualmente está ampliamente aceptado que en el caso de los priones la existencia de cepas se debe a diferencias a nivel de la estructura de la proteína prion, la cual puede adoptar conformaciones alternativas que pueden ser estables y propagarse eficientemente (Bartz *et al.*, 2000; Peretz *et al.*, 2001).

Tipificación de cepas priónicas

Dado que las herramientas genéticas no pueden ser utilizadas para la caracterización de cepas priónicas, estas deben ser diferenciadas en base a sus características fenotípicas. La tipificación o caracterización de cepas priónicas se realiza

a través de pases seriados en animales de laboratorio, fundamentalmente ratones. Tras la estabilización de la cepa en la línea murina la caracterización se realiza mediante la valoración de cuatro parámetros: el periodo de incubación, los signos clínicos, las lesiones histopatológicas producidas y el patrón inmunohistoquímico de los depósitos de PrP^{Sc} (Fraser and Dickinson, 1968; DeArmond and Prusiner, 1993). Tradicionalmente uno de los parámetros más utilizados para distinguir cepas ha sido el periodo de incubación, ya que la inoculación de distintas cepas priónicas en el mismo modelo normalmente da lugar a periodos de incubación diferenciables y reproducibles (Westaway *et al.*, 1987; Bruce, 1993). Asimismo, los patrones específicos de degeneración espongiforme y de depósito de PrP^{Sc} en el encéfalo son factores muy importantes en la caracterización de cepas priónicas. Las lesiones histopatológicas son un reflejo tanto de las variaciones en los periodos de incubación como de los signos clínicos que el individuo manifiesta durante el curso clínico de la enfermedad (Fraser, 1976). Para la evaluación de las lesiones espongiformes existe un protocolo semicuantitativo que está bien estandarizado en ratones: el perfil lesional, mediante el que se evalúa la magnitud de las lesiones producidas en nueve áreas encefálicas distintas (Fraser and Dickinson, 1968). El perfil immunohistoquímico de acumulación de PrP^{Sc} se valora de una forma similar a las lesiones espongiformes, si bien en este caso también se valora la magnitud de cada uno de los patrones de depósito de la proteína prion, clasificados en función de su morfología y localización (Jeffrey *et al.*, 2003; Gonzalez *et al.*, 2010b; Gonzalez *et al.*, 2012). El perfil immunohistoquímico ha tenido especial relevancia en la identificación del origen de distintas cepas priónicas. Este es el caso de la vECJ, la cual presenta las mismas características neuropatológicas que su cepa de origen, la EEB, lo que permitió establecer su procedencia (Bruce *et al.*, 1997; Scott *et al.*, 1999).

De este modo, se ha demostrado que la cepa infectiva es uno de los principales factores que determinan el perfil de distribución específico de las lesiones y de los depósitos de PrP^{Sc} (Bruce and Fraser, 1991). Sin embargo, el genotipo del gen *PRNP* del hospedador también juega un papel muy importante en la modulación del fenotipo neuropatológico de las EET, especialmente en lo que respecta a la distribución y la morfología de los depósitos de PrP^{Sc} en el encéfalo (Ligios *et al.*, 2002; Spiropoulos *et al.*, 2007). Ciertos polimorfismos del gen *PRNP* han demostrado ser capaces de influir en la distribución periférica de la PrP^{Sc}, hecho comprobado de forma reiterada en el scrapie ovino, ya que los animales que expresan ciertos polimorfismos asociados con la

resistencia a las EET muestran una acumulación mínima y poco frecuente de PrP^{Sc} fuera del SNC (van Keulen *et al.*, 1996; Andreoletti *et al.*, 2000; Gonzalez *et al.*, 2014b). Por un lado ciertos autores han considerado que la variabilidad en el fenotipo neuropatológico se debe fundamentalmente al genotipo del gen *PRNP* del hospedador (Spiropoulos *et al.*, 2007), sin embargo otros han asegurado que, por el contrario, este depende más bien de la cepa infectiva (Gonzalez *et al.*, 2002; Gonzalez *et al.*, 2010b). Posteriormente se afirmó que la distribución de la PrP^{Sc} dependía de interacciones complejas entre la cepa priónica y ciertos factores genéticos del hospedador (Gonzalez *et al.*, 2012).

Así pues, el periodo de incubación, los signos clínicos, las lesiones histopatológicas producidas y el patrón inmunohistoquímico de la PrP^{Sc} constituyen las principales diferencias *in vivo* que pueden observarse entre las cepas. Sin embargo, cada cepa presenta también una serie de características bioquímicas específicas que pueden utilizarse para la identificación de las mismas. Entre las más importantes se encuentran el patrón de glicosilación de la PrP^{Sc} y su movilidad electroforética tras la digestión con PK (Bessen and Marsh, 1992a; Collinge *et al.*, 1996; Parchi *et al.*, 1996; Khalili-Shirazi *et al.*, 2005), siendo actualmente uno de los principales métodos de caracterización de cepas priónicas. El patrón electroforético de una cepa priónica depende de las proporciones relativas que presenta de las formas diglicosilada, monoglicosilada y no glicosilada y del sitio de corte de la PK (Parchi *et al.*, 1996).

Como se ha mencionado, el principal método para identificar cepas priónicas es la transmisión experimental a modelos murinos. La inoculación de material infeccioso en ratones ha permitido la identificación de más de 20 fenotípicamente distintas (Bruce, 1993), la mayor parte de ellas procedentes de distintos aislados de scrapie ovino y caprino, EEB y aislados de eECJ y GSS de origen humano (Morales *et al.*, 2007). La mayor parte de las cepas de scrapie murino que se utilizan actualmente proceden de la cepa de scrapie ovino SSBP/1 (del inglés *scrapie subpassaged brain pool*). Esta proviene de un homogeneizado de tejido nervioso obtenido de tres ovejas distintas afectadas por scrapie, que fue transmitido a ovejas y cabras, realizándose más de 20 pases (Dickinson *et al.*, 1968). La transmisión de la cepa SSBP/1 a distintas líneas de ratones transgénicos ha conducido al aislamiento de 8 cepas de scrapie murino: 22C, 22A, 22M, 22L, 80V, 79A, 79V y RML (Hörnlimann *et al.*, 2007). Por otro lado, se conoce la cepa ME7, otra de las de scrapie murino más utilizadas en la investigación de las EET, que fue aislada tras el

pase en ratones de material esplénico obtenido de una oveja Suffolk infectada de forma natural por scrapie (Zlotnik and Rennie, 1963). De entre las cepas murinas no procedentes del scrapie, cabe destacar: 301C, 301V y Fukuoka, originadas las dos primeras tras la inoculación de ratones con EEB y la última con eECJ (Tateishi *et al.*, 1979; Fraser *et al.*, 1992; Bruce *et al.*, 1994). En la Figura 2 se muestra el proceso de obtención de algunas de las cepas de EET adaptadas al ratón más utilizadas actualmente en la investigación de las enfermedades priónicas.

Sin embargo, aunque se han conseguido aislar numerosas cepas en ratones, más de 20 en el caso del scrapie, la identificación de cepas de scrapie en los hospedadores naturales sigue siendo un tema controvertido, del que se sabe poco. Esto se debe a que en el caso de esta enfermedad el genotipo del gen *PRNP*, tanto del individuo donante como del receptor, puede tener un efecto significativo en el fenotipo resultante (Ligios *et al.*, 2002; Spiropoulos *et al.*, 2007), haciendo que la identificación de cepas en la oveja y la cabra sea complicado. En ovinos y caprinos se han identificado la ya mencionada SSBP/1 (Dickinson *et al.*, 1968); la cepa CH1641, la cual presenta unas características bioquímicas similares a la EEB (Foster and Dickinson, 1988); y la cepa Nor98, causante del scrapie atípico (Benestad *et al.*, 2003).

En el caso de la EEB en la vaca se han identificado tres cepas bien diferenciadas, una forma clásica (también denominada como EEB-C) y dos formas atípicas, la EEB-L y la EEB-H. Los animales afectados por EEB-L se caracterizan por la presencia de placas amiloides en el encéfalo y por mostrar un patrón de glicosilación distinto al de la EEB-C, con una banda no glicosilada de menor peso molecular (Casalone *et al.*, 2004). Por su parte, la EEB-H se caracteriza por una banda no glicosilada de mayor peso molecular que la de la EEB-C (Biacabe *et al.*, 2004). Debido al carácter zoonótico de la EEB-C, y el hecho de que se hayan detectado casos naturales de esta enfermedad en pequeños rumiantes (Eloit *et al.*, 2005; Spiropoulos *et al.*, 2011), se han desarrollado distintos métodos para diferenciar esta cepa del scrapie. Uno de los más sencillos, que aprovecha las distintas características bioquímicas de las cepas priónicas, es el uso de anticuerpos que mapeen en distintas partes de la proteína prion, como el 6H4, cuyo epítopo está localizado en el extremo C-terminal de la proteína (Korth *et al.*, 1997) , y el P4, que mapea próximo al extremo N-terminal (Shinagawa *et al.*, 1986). Tras la digestión con la enzima PK el epítopo del anticuerpo P4 se elimina total o en su mayor parte en las cepas de EEB-

C, manteniéndose intacto en el caso del scrapie, lo que permite diferenciarlas (Stack *et al.*, 2002). Un protocolo muy similar, que utiliza el anticuerpo N-terminal 12B2, se emplea también para discriminar entre distintas cepas priónicas humanas, ya que este anticuerpo no detecta, o lo hace pobremente, aquellas asociadas al patrón de PrP^{Sc} tipo 2 de la ECJ (Notari *et al.*, 2007). Por ello este anticuerpo no detecta la PrP^{Sc} de las formas MM2-cortical, MM2-talámica, VV2 ni vECJ [clasificación de Parchi y Gambetti (Parchi *et al.*, 1996)], siendo un método muy útil para diferenciar la vECJ de la mayor parte de los casos de eECJ, que presentan un patrón de glicosilación de tipo 1(Parchi *et al.*, 1999).

Finalmente, en el caso de la ECC también se han identificado distintas cepas, si bien la tipificación en este caso resulta más compleja dado que esta enfermedad afecta a animales silvestres. La transmisión experimental en ratones de material infeccioso procedente de distintas especies de cérvidos condujo al aislamiento de dos tipos de cepas diferenciables de ECC, las denominadas CWD1 y CWD2 (del inglés *chronic wasting disease*) (Angers *et al.*, 2010). Posteriormente, tras transmitir diferentes aislados procedentes de ciervos de cola blanca de distintos genotipos para el gen *PRNP* a ratones transgénicos, se aislaron dos distintas: Wisc-1 y H95+. Si bien la cepa Wisc-1 comparte muchas características con la mencionada CWD1, por lo que podría tratarse de la misma cepa, la cepa H95+ presenta características distintas a todas las anteriormente descritas, por lo que se consideró una cepa emergente de ECC. H95+ posee la capacidad de propagarse tanto en ratones *wild-type* como en ratones transgénicos que expresan un genotipo resistente a la ECC (Duque Velasquez *et al.*, 2015; Herbst et al., 2017).

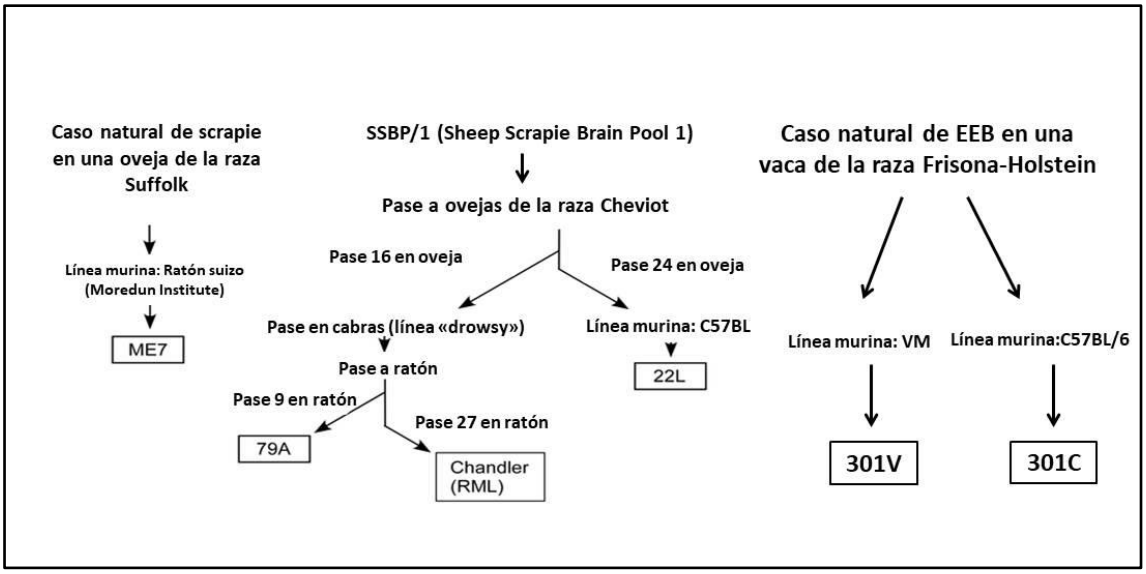

Figura 2: Esquema en el que se representa la generación de algunas de las cepas priónicas murinas más utilizadas.

LA BARRERA DE TRANSMISIÓN

La transmisión de la PrP^{Sc} entre miembros de la misma especie suele ser eficiente, produciendo en los individuos afectados signos clínicos, características neuropatológicas y periodos de incubación muy similares a los del individuo transmisor. Sin embargo, la transmisión interespecie normalmente está sujeta al fenómeno denominado "barrera de transmisión", que se manifiesta por una mayor resistencia a la propagación del prion en la nueva especie. Esta barrera se caracteriza por producir, en un primer pase de una cepa priónica a una especie, periodos de incubación extremadamente largos que en ocasiones pueden sobrepasar la esperanza de vida del nuevo hospedador. No obstante, cuando el agente es transmitido de forma sucesiva, mediante distintos pases en la nueva especie, puede producirse una adaptación del agente a la misma, y los periodos de incubación se reducen conforme la cepa alcanza la estabilidad (Priola, 1999).

Esta resistencia a la transmisión de priones de una especie a otra en un primer pase se describió por primera vez en la enfermedad de scrapie, estableciéndose el concepto de "barrera de especie" (Pattison, 1965). A este respecto se propuso que los principales determinantes de la barrera interespecie eran las diferencias entre las estructuras primarias de la PrP^{Sc} de la especie donante y la PrP^{C} de la especie hospedadora (Scott *et al.*, 1989; Prusiner *et al.*, 1990; Bartz *et al.*, 1994). Sin embargo, la cepa priónica transmitida es también un factor muy importante. Así, se sugirió que, incluso para cepas procedentes de

la misma especie, la barrera dependía en gran medida de la conformación específica de cepa de la PrP^{Sc} (Baskakov, 2014). De este modo, comenzó a utilizarse el término genérico "barrera de transmisión" para hacer referencia al conjunto de la barrera de especie y la barrera de cepa (Supattapone *et al.*, 1999; Hill *et al.*, 2000). De hecho, algunos estudios han sugerido que la barrera de transmisión está fundamentalmente modulada por las características conformacionales de la cepa transmitida, en vez de por las diferencias aminoacídicas entre las secuencias de la PrP del individuo hospedador y el transmisor (Scott *et al.*, 2005; Torres *et al.*, 2014).

Como se ha descrito, las nuevas técnicas de propagación de la PrP^{Sc} han demostrado que incluso las especies más resistentes no son completamente refractarias a la transmisión de las EET, sugiriendo que la barrera de transmisión de los priones en los mamíferos no es absoluta. En ocasiones, se ha descrito la existencia de una barrera parcial, cuando los animales inoculados presentan una enfermedad subclínica debido a una baja eficiencia de conversión de la PrP^{C} en la forma patógena (Race and Chesebro, 1998). Por otro lado, ciertas cepas priónicas, especialmente la EEB-C, tienen la capacidad de mantener sus propiedades bioquímicas y neuropatológicas al transmitirse a una nueva especie, incluso tras la realización de varios pases (Bruce *et al.*, 1997; Hill *et al.*, 1997; Torres *et al.*, 2014). Este hecho es especialmente notable en el caso del prion que causa la vECJ que, a pesar de provocar una enfermedad priónica humana, posee idénticas propiedades biológicas y bioquímicas a las del prion causante de la EEB-C, de la cual procede. Así pues, en los individuos afectados se observan las mismas características neuropatológicas que las descritas en los bovinos afectados por EEB-C (Will *et al.*, 1996; Bruce *et al.*, 1997; Hill *et al.*, 1997).

Por último, la transmisión interespecie puede dar lugar a la generación de nuevas cepas. Un ejemplo clásico es la aparición de las cepas *hyper* y *drowsy*. Estas producen en el hámster dos síndromes completamente distintos con respecto al periodo de incubación, signos clínicos y perfil lesional, y divergieron de la ETV al ser transmitida experimentalmente al hámster sirio (Bessen and Marsh, 1992b).

Factores que determinan la barrera de transmisión

Genotipo del gen PRNP

Como se ha mencionado, inicialmente se consideró que la barrera de transmisión estaba determinada únicamente por el grado de similaridad entre las secuencias de la PrP de la especie donante y la receptora. Se ha comprobado que cambios mínimos en la secuencia de la PrP^{C} de una especie pueden tener un gran impacto en la barrera de transmisión frente a una cepa específica.

Se han descrito numerosos polimorfismos del gen *PRNP* en las distintas especies. Se conocen las mutaciones puntuales, las inserciones y las deleciones. Las mutaciones puntuales producen un cambio de un nucleótido en un codón y, por lo tanto, dan lugar a un cambio aminoacídico en la proteína que se genera en la zona estructurada de esta, en la región C-terminal. Las inserciones y deleciones de nucleótidos, por otro lado, tienen lugar en la región de octapéptidos, produciendo un cambio en la región no estructurada de la PrP^{C}, en el extremo N-terminal (Goldmann, 2008). Los polimorfismos del gen *PRNP* tienen un gran impacto sobre el desarrollo de las EET de forma natural (Hunter, 1997) y pueden influir en la conversión de la PrP^{C} en la isoforma patógena PrP^{Sc} (Bossers *et al.*, 1997). Estos cambios, como se ha descrito, pueden producirse en distintas partes de la PrP^{C}, y no todos afectan en el mismo grado a la eficiencia de transmisión. Se considera que la región β2–α2 de la PrP^{C} es crítica, ya que cambios aminoacídicos mínimos en esta secuencia afectan profundamente a la susceptibilidad frente a las enfermedades priónicas (Bett *et al.*, 2012).

Los polimorfismos del gen *PRNP* ocurren de forma natural en numerosas especies y su influencia, tanto en la transmisión de priones dentro de la misma especie como entre especies distintas, ha sido ampliamente estudiada. Por ejemplo, las distintas razas ovinas presentan distintas susceptibilidades a la enfermedad de scrapie, y los polimorfismos de los codones 136, 154 y 171 del gen *PRNP* ovino juegan un papel muy importante. Así pues, el codón 136 puede codificar los aminoácidos valina (V), alanina (A) o treonina (T) (Goldmann *et al.*, 1991; Billinis *et al.*, 2004); el codón 154 codifica arginina (R), histidina (H) o leucina (L) (Goldmann *et al.*, 1991; Alvarez *et al.*, 2006) y el 171 puede codificar arginina, histidina, glutamina (Q) o lisina (K) (Goldmann *et al.*, 1990; Belt *et al.*, 1995; DeSilva *et al.*, 2003). Así, las ovejas que expresan los alelos VRQ o ARQ presentan una gran vulnerabilidad al scrapie clásico, mientras que la expresión del alelo ARR otorga

resistencia (Belt *et al.*, 1995). Además, el haplotipo ARR posee un efecto dominante, y tanto los animales homocigotos como heterocigotos presentan un menor riesgo a padecer esta enfermedad priónica (Goldmann *et al.*, 1994; Westaway *et al.*, 1994b; Clouscard *et al.*, 1995).

En los cérvidos también se han descrito numerosos polimorfismos que están relacionados con la susceptibilidad o resistencia a la ECC. Así, un polimorfismo en el codón 132 del gen *PRNP* produce un cambio de metionina (M) a leucina, cambio descrito como protector frente a la ECC en el alce de las Montañas Rocosas (*Cervus elaphus nelsoni*) (O'Rourke *et al.*, 1999). En el ciervo de cola blanca (*Odocoileus virginianus*) los dos polimorfismos más estrechamente relacionados con la resistencia a la enfermedad parecen ser el cambio de glutamina por histidina y de glicina (G) por serina (S) en los codones 95 y 96 del gen *PRNP* respectivamente. Estudios epidemiológicos demostraron que la incidencia de la enfermedad es significativamente menor en los ciervos que expresan al menos una copia de estos alelos (Johnson *et al.*, 2003; O'Rourke *et al.*, 2004; Johnson *et al.*, 2006). Posteriormente se corroboró el efecto protector de los polimorfismos Q95H y G96S mediante la realización de un bioensayo en el que se demostró que los ciervos que expresaban al menos una copia de estos alelos presentaban un periodo de incubación de la ECC mucho más largo que los animales control (Johnson *et al.*, 2011). El cambio de serina por fenilalanina (F) del ciervo mula (*Odocoileus hemionus*) también ha sido asociado con la resistencia a esta enfermedad, pues los animales 225SF o 225FF presentan incidencias de ECC mucho más bajas y periodos de incubación significativamente más largos que los ciervos 225SS (Jewell *et al.*, 2005; Wolfe *et al.*, 2014).

El polimorfismo en el residuo 129 (M o V) de la PrP humana también tiene un efecto similar sobre la susceptibilidad a las EET. Se ha demostrado que la homocigosis del codón 129 es un factor predisponente para el desarrollo de las formas esporádicas y adquiridas de la ECJ, así como para el kuru, mientras que la incidencia de estas enfermedades es mucho menor en los heterocigotos (Palmer *et al.*, 1991; Lee *et al.*, 2001; Brandel *et al.*, 2003). El efecto de este polimorfismo parece ser aún mayor en el caso de la vECJ, ya que todos los casos confirmados de esta enfermedad corresponden a individuos homocigotos para metionina en el codón 129 (Will *et al.*, 2000), con una excepción en un caso subclínico detectado en un individuo heterocigoto (Bishop *et al.*, 2013). Este hecho parece correlacionarse con los resultados obtenidos en transmisiones

experimentales de EEB a ratones transgénicos. Se ha demostrado que la expresión de valina en el codón 129 actúa como una barrera de transmisión para la EEB, produciendo alteraciones en el fenotipo de la enfermedad (Wadsworth *et al.*, 2004; Fernandez-Borges *et al.*, 2017a). La heterocigosis para otro polimorfismo diferente del gen *PRNP* humano, el E219K, (E= ácido glutámico) también está asociada con la resistencia a la eECJ, lo cual ha sido observado en la población japonesa (Shibuya *et al.*, 1998). Por último, el polimorfismo humano G127V es una variante que se detectó entre los individuos que no se infectaron durante la epidemia de kuru, considerando que se trataba de un factor genético de resistencia que se seleccionó como consecuencia de esta (Mead *et al.*, 2009). Se ha demostrado que este polimorfismo proporciona una gran resistencia frente a las enfermedades priónicas humanas y que los mecanismos por los que produce este efecto de resistencia son probablemente distintos a los producidos por el polimorfismo M129V (Asante *et al.*, 2015).

Otro ejemplo de cómo ciertos cambios en la secuencia de la PrP^C pueden afectar a la barrera de transmisión para las enfermedades priónicas es el ya mencionado *bank vole*. Se considera que la expresión de isoleucina en el codón 109 en esta especie es la clave de su excepcional susceptibilidad a las EET, ya que se cree que este cambio hace que su PrP^C sea inestable (Cartoni *et al.*, 2005; Watts *et al.*, 2012; Watts *et al.*, 2014).

Como se ha mencionado, los cánidos parecen ser los mamíferos más resistentes a la transmisión de las EET. Se propuso que esta resistencia pudiera deberse a ciertos residuos aminoacídicos presentes en la PrP^C canina. La PrP^C de los cánidos es polimórfica en tres posiciones: G101S, D163E y T196I. Así pues, en el codón 163, los cánidos expresan ácido aspártico (D) o ácido glutámico mientras que el resto de especies de mamíferos expresan asparagina (N) (Stewart *et al.*, 2012). Se ha demostrado que esta sustitución juega un papel fundamental en la resistencia de estas especies a las EET ya que los ratones transgénicos que expresan exclusivamente este cambio aminoacídico (D158 en la PrP del ratón) son completamente resistentes a la inoculación con diversas cepas priónicas (Fernandez-Borges *et al.*, 2017b).

Ciertos cambios aminoacídicos en la PrP^C tienen la capacidad, no sólo de proporcionar resistencia a las EET a los individuos que los expresan, sino también de bloquear el malplegamiento de la PrP^C endógena (la denominada como "de tipo silvestre" o "*wild-type*") cuando la PrP^C con el cambio es co-expresada en el mismo individuo. Este

efecto se conoce como inhibición dominante negativa y ha sido ampliamente estudiado en cultivos celulares. Se han identificado varias moléculas que ejercen este efecto, siendo algunas de ellas PrP con cambios aminoacídicos que se producen de forma natural en algunas especies. Este es el caso de las PrP con las sustituciones Q171R, E219K y G127V que, como se ha descrito, proporcionan resistencia frente a las EET en la oveja y en el ser humano. Se ha demostrado que, cuando estas proteínas mutadas se co-expresan con la PrP^{C} del ratón, no sólo no se malpliegan, sino que son capaces de bloquear el malplegamiento de la PrP^{C} endógena del ratón y por tanto impiden la propagación de priones tanto en cultivos celulares como en ratones transgénicos (Kaneko *et al.*, 1997b; Zulianello *et al.*, 2000; Perrier *et al.*, 2002; Asante *et al.*, 2015).

La cepa priónica

La barrera de transmisión también está fuertemente influenciada por la cepa priónica transmitida. Un ejemplo claro es la capacidad de la EEB-C para transmitirse fácilmente a un gran número de especies sin sufrir grandes alteraciones en sus propiedades bioquímicas o neuropatológicas (Collinge *et al.*, 1996; Torres *et al.*, 2014). Por el contrario, se observó que existía una fuerte barrera de transmisión frente a otras cepas priónicas no relacionadas con la EEB que presentaban una secuencia aminoacídica idéntica a esta (Torres *et al.*, 2014). Por tanto, está demostrado que las diferencias entre las secuencias de aminoácidos de la PrP^{Sc} del inóculo y la PrP^{C} del huésped son insuficientes para explicar el fenómeno de la barrera de transmisión. Asimismo, ciertas cepas de scrapie se transmiten de forma efectiva a ratones transgénicos que expresan la PrP^{C} bovina, sin que los periodos de supervivencia se reduzcan significativamente en pases subsiguientes (Scott *et al.*, 2005).

De este modo, actualmente se considera que tanto la cepa priónica como la secuencia de la PrP^{C} del hospedador son los dos principales determinantes de la barrera de transmisión (Collinge, 2001).

La ruta de infección

La vía por la que los priones entran al organismo también es un factor que puede afectar en gran medida a la magnitud de la barrera de transmisión. Las infecciones naturales generalmente se producen por vía oral, una ruta que normalmente es mucho menos eficiente que la inoculación intracerebral (Kimberlin and Walker, 1988).

Asimismo, en ciertas inoculaciones experimentales se ha demostrado que el título infeccioso que se alcanza en el encéfalo cuando la enfermedad se produce tras una infección intracerebral es mucho más alto que el alcanzado tras una inoculación parenteral (Kimberlin and Walker, 1978). Sin embargo, se ha observado que las ovejas inoculadas con scrapie por distintas vías presentan un perfil de depósito de PrP^{Sc} idéntico, tanto en el encéfalo como en los tejidos periféricos, por lo que la ruta de entrada del prion parece no afectar a la patogenia de la enfermedad en gran medida cuando animales del mismo genotipo son inoculados con la misma cepa priónica (Gonzalez *et al.*, 2014a). Otros estudios parecen indicar que el efecto de la ruta de infección parece influir en la duración del periodo de replicación del prion en el encéfalo, es decir, en el tiempo que transcurre entre que la PrP^{Sc} empieza a ser detectable en el encéfalo y la aparición de la enfermedad clínica. Tras la inoculación de la cepa 263K en hámsteres por distintas vías se observó que el periodo de replicación de la PrP^{Sc} más corto se producía en los animales inoculados por vía intraperitoneal, seguido de los animales inoculados por vía intracerebral y, finalmente, los inoculados por vía intraocular, que presentaban el periodo más largo. Este efecto se atribuyó a las diferentes eficiencias con las que una cepa priónica determinada alcanza las áreas del encéfalo por las que tiene mayor tropismo. Esta eficiencia puede variar en función de las vías neuronales por las que la PrP^{Sc} alcanza el encéfalo y, por tanto, puede variar en función de la vía de inoculación (Kimberlin and Walker, 1986).

Otros factores implicados en la conversión de la PrP^{C} en la forma patógena y su implicación en la barrera de transmisión: La glicosilación de la PrP^{C}

Como se ha mencionado anteriormente, la PrP^{C} puede estar presente en el organismo en tres glicoformas distintas, la diglicosilada, la monoglicosilada y la no glicosilada. La PrP^{C} del ratón contiene dos sitios susceptibles de ser glicosilados mediante la adición de N-glicanos, en las posiciones N180 y N196. La distribución relativa de formas di-, mono- y no glicosiladas de la PrP^{C} varía entre las especies, e incluso se han identificado variaciones regionales en la estequiometría de las distintas glicoformas en el encéfalo de un mismo hospedador (DeArmond *et al.*, 1999; Beringue *et al.*, 2003; Somerville *et al.*, 2005). Inicialmente se sugirió que los niveles de glicosilación de la PrP^{C} podrían estar relacionados con su capacidad de conversión en la forma patógena PrP^{Sc}, ya que la glicosilación de la PrP^{C} afecta profundamente a los patrones de depósito de la PrP^{Sc} y ambas formas difieren con respecto al contenido en glicanos (DeArmond *et al.*, 1997; Rudd *et al.*, 1999). Ya se ha descrito el papel del genotipo del gen *PRNP* y, por

tanto, de la secuencia de la PrP^{C} en la barrera de transmisión. El estudio de la posible influencia de la glicosilación surgió con el objetivo de conocer si las modificaciones post-traduccionales de la PrP también eran determinantes en la barrera interespecie, como lo es la secuencia primaria de la proteína.

Experimentos *in vitro* demostraron que cuando la PrP^{C} y la PrP^{Sc} proceden de especies distintas la glicosilación de la PrP^{C} puede afectar significativamente a la formación de la PrP^{Sc} al modular las interacciones entre ambas proteínas. Por ello, se sugirió que los glicanos unidos a la PrP^{C} podrían ser determinantes en la transmisión de priones entre distintas especies (Priola and Lawson, 2001). Estudios posteriores, realizados *in vivo*, sugirieron que, si bien la glicosilación de la PrP no es indispensable para que la infección priónica se produzca (Tuzi *et al.*, 2008; Cancellotti *et al.*, 2013), sí posee una gran influencia sobre la eficiencia de transmisión intra- e interespecie de los priones (Neuendorf *et al.*, 2004; Tuzi *et al.*, 2008; Wiseman *et al.*, 2015). Además, otros estudios propusieron que la glicosilación de la PrP^{C} del huésped también podría ser un factor determinante de las características fenotípicas de las cepas priónicas (Cancellotti *et al.*, 2013). Sin embargo, otros autores han sugerido que la interpretación de estos resultados debe realizarse con cautela, pues en estos estudios generalmente se utilizan modelos murinos transgénicos a los cuales se les insertan mutaciones en la secuencia de la PrP^{C} con el fin de eliminar los sitios de glicosilación, mutaciones que podrían ser en parte responsables de las alteraciones observadas en la barrera de transmisión y en las propiedades de las cepas priónicas transmitidas (Salamat *et al.*, 2011; Moudjou *et al.*, 2016). De hecho, se ha demostrado que las moléculas no glicosiladas de PrP^{Sc}, generadas *in vitro*, son capaces de mantener sus características neuropatológicas y bioquímicas específicas de cepa al ser transmitidas a ratones que expresan una PrP^{C} normalmente glicosilada, lo cual condujo a la conclusión de que los glicanos unidos a la PrP^{C} no son esenciales en la determinación de las propiedades de las cepas priónicas (Piro *et al.*, 2009; Moudjou *et al.*, 2016). Por ello, el papel específico que juegan los glicanos unidos a la PrP^{C} en la transmisión y en la modulación de las características patobiológicas de las cepas priónicas sigue siendo un tema controvertido hoy en día.

PATOGENIA Y TRANSMISIÓN

Las enfermedades priónicas pueden tener un origen esporádico, hereditario o bien ser adquiridas como resultado de una infección. Aunque todas ellas poseen un evento patogénico común, la conversión de la PrP^{C} en la isoforma anómala PrP^{Sc}, muchos de los mecanismos implicados en la patogenia de las EET siguen sin conocerse con exactitud, especialmente en el caso de las enfermedades priónicas espontáneas, cuyo origen es desconocido.

Patogenia y transmisión de las EET adquiridas de forma natural

Este grupo de enfermedades está constituido por todas aquellas EET producidas por la exposición del individuo a priones procedentes de una fuente exógena, presente en el entorno. El scrapie, la ECC, la EEB y la vECJ constituyen las formas de EET adquiridas de forma natural más relevantes, y existen evidencias sustanciales de que, en la mayoría de los casos, estas enfermedades están producidas por la ingestión de priones y la subsiguiente invasión del organismo a través del tracto alimentario (Beekes and McBride, 2007). Si bien las citadas son las EET adquiridas de mayor relevancia, también se incluyen en este grupo la EEF, la EUE y el kuru (Gajdusek, 1977; Beekes and McBride, 2007), y probablemente también la ETV (Hartsough and Burger, 1965; Marsh and Bessen, 1993). La patogenia de las distintas EET adquiridas presenta propiedades específicas en función de la enfermedad y el huésped. La ruta seguida por los priones en el organismo, cuando estos acceden por vía oral, se realiza en cuatro fases características, que pueden ocurrir en ocasiones simultáneamente:

- Acumulación del agente infeccioso en el tejido linfoide.
- Diseminación al sistema nervioso periférico.
- Llegada del prion al SNC y diseminación en el mismo.
- Propagación centrífuga desde el SNC a tejidos periféricos.

En la patogenia de ciertas enfermedades priónicas, especialmente en el caso del scrapie, también parece existir una diseminación hematógena del prion, que podría actuar como una ruta complementaria de neuroinvasión (Siso *et al.*, 2010). En el scrapie, la ingestión de placentas contaminadas parece jugar un papel muy importante en la transmisión de la enfermedad (Brotherston *et al.*, 1968; Dickinson *et al.*, 1974), aunque la infección percutánea y otras vías de menor importancia también podrían contribuir en

la entrada de priones en la especies ovina y caprina (van Keulen *et al.*, 2002). De este modo, la transmisión natural del scrapie clásico parece ocurrir principalmente por vía horizontal, por contacto directo entre los animales o de forma indirecta debido a la contaminación del ambiente (Hoinville, 1996). Aunque la transmisión materna del scrapie está reconocida en condiciones naturales, es difícil de evaluar debido a la posible transmisión lateral tras el nacimiento. Sin embargo, existen evidencias experimentales de la transmisión del scrapie *in utero* (Hourrigan *et al.*, 1979; Garza *et al.*, 2011; Foster *et al.*, 2013), aunque esta depende en gran medida del genotipo del gen *PRNP* tanto de la madre como del feto (Andreoletti *et al.*, 2002; Tuo *et al.*, 2002). Junto con el scrapie, la ECC es la otra EET considerada no solo transmisible, sino epidémica o contagiosa. Además, se ha demostrado que los priones causantes de la ECC se eliminan al medio en cantidades significativas a través de secreciones y excreciones, como la saliva, la orina y las heces, pudiendo tener gran importancia en la contaminación del medio y en la propagación de la enfermedad (Mathiason *et al.*, 2006; Haley *et al.*, 2009; Tamguney *et al.*, 2009). Por otro lado, en el caso de la EEB no existen evidencias de que la propagación horizontal o la transmisión vertical existan en condiciones naturales (Curnow and Hau, 1996; Wrathall *et al.*, 2002) y actualmente está aceptado que la epidemia de la EEB se produjo por la intervención humana, ya que el ganado bovino fue alimentado con harinas de carne y hueso obtenidas de rumiantes afectados por EET.

Cuando el prion penetra por vía oral, la invasión del organismo se produce generalmente a través del tejido linfoide asociado al intestino, principalmente a nivel del íleon. En este proceso participan enterocitos modificados denominados células M, que captan la PrP^{Sc} y la incorporan al tejido linfoide subepitelial, donde se acumula y replica en los macrófagos de cuerpo tingible y en las células dendríticas foliculares (van Keulen *et al.*, 1996; Jeffrey *et al.*, 2001; Mabbott and Bruce, 2001). Otro punto muy importante de entrada del agente al organismo es la tonsila palatina (Mabbott and Bruce, 2001). Así, generalmente en la fase preclínica de la enfermedad, la PrP^{Sc} puede estar presente en el tejido linfoide, por lo que en el caso del scrapie puede realizarse un diagnóstico temprano mediante la obtención de biopsias de la tonsila palatina, del tercer párpado, del linfonodo retrofaríngeo o de la mucosa rectal (Monleon *et al.*, 2005; Langeveld *et al.*, 2006; Vargas *et al.*, 2006; Gonzalez *et al.*, 2008). De un modo similar, los primeros lugares en los que puede detectarse el agente causal de la ECC son los tejidos linfoides asociados al tracto alimentario, la tonsila, las Placas de Peyer y los linfonodos retrofaríngeos, siendo estos

últimos generalmente el tejido de elección para el diagnóstico de esta enfermedad (Sigurdson *et al.*, 1999).

Tras un tiempo de permanencia en el sistema linforreticular, el prion se dirige al SNC. En el scrapie el proceso de neuroinvasión subsiguiente puede variar enormemente en función de la cepa priónica implicada y de la susceptibilidad genética del individuo. De una forma similar a lo que ocurre en la EEB, en la que la implicación del sistema linforreticular es mínima (Buschmann and Groschup, 2005), los ovinos de genotipo resistente al scrapie clásico apenas acumulan PrP^{Sc} en los tejidos linfoides (van Keulen *et al.*, 1996; Andreoletti *et al.*, 2000; Houston *et al.*, 2002; Jeffrey *et al.*, 2002; Ersdal *et al.*, 2003). Este efecto parece ser debido a una modulación de la patogenia ejercida por ciertos polimorfismos, siendo la expresión de arginina en el codón 171 el que parece tener un mayor impacto (Gonzalez *et al.*, 2010a; Gonzalez *et al.*, 2014b). La influencia del genotipo para el gen *PRNP* sobre la distribución del prion también se ha descrito en la ECC, ya que los ciervos mula de genotipo 225SF presentan una menor y más limitada acumulación de PrP^{CWD} en distintos tejidos que los animales de genotipo 225SS a idénticos intervalos post-inoculación (Fox *et al.*, 2006). También se ha observado un efecto similar en los ciervos de cola blanca que expresan el alelo S96, relacionado con la resistencia (Johnson *et al.*, 2006; Hoover *et al.*, 2017). Sin embargo, en ambos casos, se ha considerado que el efecto de estos polimorfismos sobre la distribución del prion se debe más bien a que dan lugar a un desarrollo más lento de la enfermedad, más que a una alteración de la patogenia.

El proceso de neuroinvasión parece comenzar con una transferencia del prion desde el sistema linforreticular al sistema nervioso entérico desde donde se extiende al sistema nervioso autónomo, pudiendo llegar la PrP^{Sc} al SNC a través de dos puntos principales: la médula espinal a nivel torácico y el núcleo motor dorsal del nervio vago, en la médula oblongada (Beekes *et al.*, 1998; Beekes and McBride, 2000). Por ello, la médula oblongada es la porción encefálica que se usa como muestra de elección en los actuales métodos de diagnóstico del scrapie y la EEB, si bien es cierto que en el scrapie atípico el prion se localiza fundamentalmente a nivel del cerebelo (Benestad *et al.*, 2008). El núcleo motor dorsal del nervio vago también es la primera zona del encéfalo en la que se deposita el prion en el caso de la ECC cuando la infección se produce por vía oral (Seelig *et al.*, 2010). Tras la llegada al SNC, el prion se disemina por el mismo (Kimberlin and Walker, 1980) acumulándose en mayor o menor medida en determinadas áreas

encefálicas, lo cual varía en función de la cepa priónica, del periodo de incubación y de la especie del animal (Fraser and Dickinson, 1968; Prusiner, 1998b; Ayers *et al.*, 2009). A medida que el prion se disemina en el SNC aparecen la neurodegeneración, la vacuolización del tejido encefálico y la gliosis, produciéndose los signos clínicos típicos de las enfermedades priónicas. Asimismo, se acepta en la actualidad que en ciertas EET la vía hematógena también podría actuar como una ruta de neuroinvasión complementaria a nivel de los órganos circunventriculares, en donde no existe barrera hematoencefálica (Siso *et al.*, 2009; Siso *et al.*, 2010) .

Patogenia y transmisión de las EET espontáneas y familiares

En el caso de las enfermedades priónicas de origen familiar, y, especialmente en el caso de las de origen esporádico, existen muchas más incógnitas con respecto a los mecanismos patogénicos responsables de su aparición. El primer caso de Creutzfeldt-Jakob de origen familiar fue registrado en 1924 (Kirschbaum, 1924), sin embargo, fue en 1930 cuando se demostró que este individuo pertenecía a la gran familia Backer (Meggendorfer, 1930). Subsiguientemente se comprobó que esta familia era portadora de un polimorfismo asociado con la fECJ (Kretzschmar *et al.*, 1995). Años más tarde se corroboró la transmisibilidad tanto de la fECJ como de la eECJ, lo que constituyó un avance importante en el estudio de las formas esporádicas y genéticas de las EET, ya que se demostró que las enfermedades priónicas tenían propiedades únicas, pues podían ser al mismo tiempo hereditarias e infecciosas (Gajdusek *et al.*, 1968; Gibbs *et al.*, 1968; Masters *et al.*, 1981). Así, se considera que la transmisión de las formas familiares de las EET se debe a que las PrP^{C} portadoras de mutaciones poseen una gran propensión a convertirse espontáneamente a la forma patógena (Prusiner, 1989; Gambetti *et al.*, 2003). Como se ha mencionado, el origen de las EET esporádicas es desconocido, y se han propuesto numerosas teorías con el fin de explicar cómo aparecen este tipo de enfermedades priónicas. Entre otras se ha sugerido que las EET esporádicas son consecuencia de mutaciones somáticas espontáneas del gen *PRNP* o de cambios conformacionales estocásticos en la estructura de la PrP^{C} (Prusiner, 2001). De acuerdo con otras hipótesis, en el encéfalo existe una pequeña cantidad de isoformas proteicas similares a la PrP^{Sc} , las cuales podrían estar ligadas a otras proteínas que las mantienen inactivas, apareciendo las EET esporádicas cuando este mecanismo protector falla a consecuencia del envejecimiento (Safar *et al.*, 2005; Yuan *et al.*, 2006). Finalmente, se

ha sugerido que al menos ciertos casos de eECJ se deben a una exposición encubierta y de bajo nivel a un "factor externo común"(Linsell *et al.*, 2004; Safar, 2012).

Neurodegeneración

El proceso de neurodegeneración que se produce en las EET comprende multitud de acontecimientos que se desarrollan secuencial o simultáneamente en el SNC, como son la acumulación de agregados proteicos, la degeneración espongiforme, las alteraciones sinápticas, la neuroinflamación y la muerte neuronal (Budka, 2003). Los mecanismos moleculares implicados en la neurodegeneración no se conocen en su totalidad, sin embargo, no existe duda con respecto a que la formación y la acumulación progresiva de la PrP^{Sc} en el encéfalo es el hecho desencadenante de la enfermedad. No obstante, el mecanismo patogénico a través del que la PrP^{Sc} produce las EET es, en su mayor parte, desconocido. Se ha propuesto que las enfermedades priónicas podrían originarse como resultado de la pérdida de una función crítica de la PrP^{C}, que resulta en neurodegeneración. Sin embargo, los ratones *knock-out* para el gen *PRNP* no desarrollan ningún tipo de enfermedad, lo que parece indicar que estas enfermedades no se producen por una pérdida de la función fisiológica de la PrP^{C}, sino más bien por una actividad tóxica ejercida por parte de la PrP^{Sc} (Hetz *et al.*, 2003a). Esta es la teoría más aceptada en la actualidad, sobre todo teniendo en cuenta que muchas otras enfermedades neurodegenerativas como la enfermedad de Alzheimer, Párkinson, esclerosis lateral amiotrófica o enfermedad de Huntington también están causadas por la acumulación de proteínas malplegadas en el encéfalo(Carrell and Lomas, 1997; Martin, 1999). Asimismo, se ha comprobado la toxicidad directa de estas proteínas malplegadas en cultivos neuronales (Soto, 2003).

Se han propuesto varios mecanismos neuronales como factores críticos determinantes del desarrollo de las EET. Entre éstos cabe mencionar el posible papel que desempeña el estrés del retículo endoplásmico (RE) y la posible inhibición de la degradación proteica mediada por el sistema ubiquitino-proteasómico (UPS).

Mecanismos neuronales implicados en la neurodegeneración: El estrés del retículo endoplásmico y la inhibición del sistema ubiquitino-proteasómico

La proteostasis es un proceso homeostático mediante el cual la célula regula la cantidad y la localización de las proteínas. Ya que las proteínas pueden acumularse y afectar a la función celular normal, todas las células poseen la capacidad de degradar proteínas dañadas, malplegadas o no necesarias. Este control de calidad requiere de la acción coordinada de las chaperonas y de los sistemas proteolíticos (Andre and Tabrizi, 2012).

El plegamiento de las proteínas en la célula ocurre fundamentalmente en el citosol y en el retículo endoplásmico (RE), sin embargo el plegamiento proteico en el RE es un proceso más complejo ya que en éste orgánulo las proteínas son modificadas tras la traducción como en el caso de la PrP^{C}, mediante la adición de N-glicanos o la formación de puentes disulfuro (Schroder and Kaufman, 2005).

En el RE existen tres grandes grupos de proteínas encargadas del plegamiento proteico: las lectinas, las foldasas y las chaperonas moleculares. Las lectinas colaboran en el control de calidad y la degradación de glicoproteínas (Schroder and Kaufman, 2005). Las foldasas catalizan ciertos procesos básicos de plegamiento y entre ellas destacan las peptidil-prolil cis-trans isomerasas (PPI) y las proteína disulfuro-isomerasas (PDI) (Schroder and Kaufman, 2005; Mays and Soto, 2016). En el ser humano se han descrito al menos 21 tipos de PDI (Benham, 2012), sin embargo la mejor caracterizada es la PDIA1 (que en ocasiones se denomina solo como PDI) (Ge *et al.*, 2013). La PDI cataliza la formación, rotura y reorganización de los puentes disulfuro de las proteínas a medida que estas se pliegan en el RE (Ge *et al.*, 2013) pero también se comporta como una chaperona molecular, contribuyendo al correcto plegamiento de las proteínas y evitando su agregación (Freedman *et al.*, 1994; Wang, 1998; Wilson *et al.*, 1998; Bottomley *et al.*, 2001). La PDI se encuentra principalmente en el interior del RE, en altas concentraciones (Lyles and Gilbert, 1991) y se ha demostrado que juega un papel fundamental en numerosos procesos patológicos (Benham, 2012).

Con respecto a las enfermedades priónicas se ha demostrado que las proteínas de la familia PDI se sobreexpresan en el encéfalo de pacientes afectados por vCJD y sCJD (Yoo *et al.*, 2002; Hetz *et al.*, 2003b; Torres *et al.*, 2015) y en roedores infectados con distintas cepas priónicas (Hetz *et al.*, 2005; Wang *et al.*, 2012). Además se ha demostrado

que la sobreexpresión de la PDI comienza en estadios tempranos de la enfermedad y aumenta de forma continua hasta la fase terminal (Wang *et al.*, 2012), sugiriéndose, dadas las funciones de estas proteínas, que su sobreexpresión es un intento del organismo para corregir el plegamiento anómalo de la PrP^{Sc} e incrementar su eliminación (Yoo *et al.*, 2002; Hetz *et al.*, 2003b; Hetz *et al.*, 2005). Mediante el uso de cultivos celulares se observó que la Grp58, también denominada PDIA3, interactuaba con la PrP y que actuaba como un factor neuroprotector en las EET (Hetz *et al.*, 2005). Sin embargo, en un modelo celular de la enfermedad de Huntington, se demostró que la PDI también podía inducir la apoptosis, desencadenando una cascada de caspasas y la muerte celular (Hoffstrom *et al.*, 2010) . Finalmente se comprobó que la PDI juega un papel complejo durante el curso de las enfermedades priónicas, comportándose como un factor protector en estadios iniciales mediante la eliminación de proteínas malplegadas, mientras que en estadios terminales, debido a la perturbación crónica de la homeostasis del RE, lo cual conduce a la S-nitrosilación de la PDI, induce la apoptosis celular (Wang *et al.*, 2012).

Por otro lado, las chaperonas moleculares son un conjunto de proteínas cuyas principales funciones son favorecer el plegamiento de proteínas recién formadas y facilitar su tránsito entre compartimentos celulares, así como ayudar al correcto plegamiento de las mismas cuando éstas sufren un proceso de desnaturalización (Large *et al.*, 2009; Waters *et al.*, 2009; Wong and Cuervo, 2010; Koga *et al.*, 2011). Se ha sugerido que la chaperona molecular BiP (proteína de unión a inmmunoglobulina), que pertenece a la familia de proteínas Hsp70 (heat shock proteins o proteínas de choque térmico de 70 kilodaltons) juega un papel fundamental en el plegamiento y la maduración de la PrP^{C}, siendo importante para la degradación de ciertas formas mutadas de la PrP (Jin *et al.*, 2000). Asímismo, también se ha descrito la acumulación de estas proteínas en ciertas poblaciones celulares en el scrapie natural (Serrano *et al.*, 2011).

Así, las chaperonas identifican proteínas anormales y contribuyen a que estas proteínas recuperen la estabilidad. Sin embargo, si la reparación no es posible, estas proteínas dañadas o no necesarias son eliminadas para evitar su acumulación (Wong and Cuervo, 2010). Las dos principales vías de eliminación de proteínas y organelas en las células eucariotas son el sistema ubiquitino-proteasómico y la degradación lisosomal a través de la autofagia (Ciechanover, 2005; Gomes *et al.*, 2006).

Si bien la patogenia de las enfermedades priónicas no se conoce con exactitud, se ha sugerido que la perturbación en la homeostasis del RE inducida por la acumulación de PrP^{Sc} puede conducir a la muerte neuronal y la neurodegeneración (Hetz and Glimcher, 2009; Torres *et al.*, 2010). Cuando la homeostasis del RE se altera debido a la continua acumulación de proteínas malplegadas en su interior, produciéndose el llamado "estrés del RE", se inicia una respuesta denominada respuesta a proteínas desplegadas (*unfolding protein response*, UPR), en la cual se produce una sobreexpresión de chaperonas moleculares y foldasas, como la BiP y la PDI, para tratar de recuperar el equilibrio (Turano *et al.*, 2002; Rutkowski *et al.*, 2008; Haefliger *et al.*, 2011). La UPR es una respuesta celular destinada a mejorar el plegamiento de proteínas en el RE, disminuir la tasa de producción de proteínas y su translocación en el interior de este orgánulo y aumentar la eliminación de proteínas malplegadas a través de la vía de degradación asociada al retículo endoplásmatico (*Endoplasmic-reticulum-associated protein degradation*, ERAD) (Zhang and Kaufman, 2006). Esta vía de degradación marca estas proteínas malplegadas o dañadas para que sean eliminadas a través del sistema ubiquitino-proteasómico (UPS). El UPS cataliza la eliminación de proteínas malplegadas, siendo un mecanismo fundamental de control post-translacional, y regula los niveles de muchas proteínas de vida corta, como las proteínas implicadas en la división celular, la transcripción de genes o la endocitosis (Hochstrasser, 1995; Glickman and Ciechanover, 2002; Gomes *et al.*, 2006; Zheng *et al.*, 2009).

El UPS está formado por el proteasoma (también denominado proteosoma), una compleja red de enzimas y una pequeña proteína de 8.5 kDa denominada ubiquitina. El proteasoma es un complejo proteico que se encuentra en el núcleo y el citoplasma de las células eucariotas y presenta un núcleo de 20S y dos subunidades reguladoras 19S (Peters *et al.*, 1994). Las enzimas marcan con ubiquitina las proteínas que requieren ser destruidas, formándose normalmente una cadena poliubiquitínica. Estas proteínas ligadas a las cadenas poliubiquitínicas son posteriormente reconocidas y degradadas por el proteasoma (Coux *et al.*, 1996; Hershko and Ciechanover, 1998). Cuando la degradación por parte del UPS no es capaz de recuperar la homeostasis celular, la principal ruta alternativa para la eliminación de proteínas es, como se ha comentado, la autofagia a través de los lisosomas (Zheng *et al.*, 2009). Sin embargo, cuando el estrés del RE persiste debido a la acumulación de proteínas y ni la UPR ni la autofagia consiguen recuperar el equilibrio, la célula se dirige hacia la apoptosis. En la figura 3 se representa un esquema

del proceso. Si bien los mecanismos apoptóticos desencadenados por el estrés del RE no se conocen en su totalidad, sí se sabe que se activan dos rutas principales: una mediada a través de factores de transcripción y otra mediada a través de las caspasas (Szegezdi *et al.*, 2003). La primera de estas rutas lleva a una alteración del balance Bcl2/Bax(Wang *et al.*, 1998) . La segunda ruta, mediada por las caspasas, es independiente de las mitocondrias y se inicia con la activación de la caspasa-12, una caspasa residente en el RE (Nakagawa *et al.*, 2000; Hitomi *et al.*, 2004).

Existe un grupo diverso de enfermedades neurodegenerativas que se caracterizan por la presencia de depósitos intracelulares de proteínas mal conformadas. Se cree que las alteraciones en el funcionamiento del UPS pueden contribuir a la aparición de estas enfermedades, ya que los agregados de proteínas parecen resistir la degradación proteasómica (Bence *et al.*, 2001; Sherman and Goldberg, 2001). Así pues, el deterioro de la función del UPS se ha asociado con la etiología de la enfermedad de Alzheimer (Lam *et al.*, 2000), Párkinson (Cook and Petrucelli, 2009), esclerosis lateral amiotrófica (Cheroni *et al.*, 2009) y de la enfermedad de Huntington (Seo *et al.*, 2004).

En el caso de las enfermedades priónicas se ha demostrado que se produce estrés del RE, dando lugar a la ya mencionada sobreexpresión de foldasas y chaperonas que se ha observado en pacientes humanos, (Yoo *et al.*, 2002; Hetz *et al.*, 2003b; Torres *et al.*, 2015) en casos de EEB (Tang *et al.*, 2010) y en modelos experimentales inoculados (Hetz *et al.*, 2005; Wang *et al.*, 2012). Se ha comprobado también que el estrés prolongado del RE produce una acumulación de PrP no translocada en el citosol (Kang *et al.*, 2006; Orsi *et al.*, 2006), que podría agregarse e inhibir la actividad del proteasoma (Kristiansen *et al.*, 2007; Quaglio *et al.*, 2011). Asimismo se demostró, mediante el uso de cultivos celulares, que la proteína prión patológica es capaz de inhibir específicamente las subunidades proteolíticas β del proteasoma, que constituyen los sitios activos de las proteasas (Kristiansen *et al.*, 2007). El papel del UPS en la patogenia de las EETs también ha sido estudiado *in vivo* ya que en el año 2003 se generó un ratón transgénico que permitía evaluar la actividad del sistema ubiquitino-proteasómico *in vivo*, la línea transgénica Ub^{G76V}-GFP/1 (Lindsten *et al.*, 2003). Estos ratones expresan una ubiquitina marcada con una proteína verde fluorescente, la *green fluorescent protein* (GFP) que es sustrato del proteasoma. El deterioro del sistema ubiquitino-proteasómico da lugar a la acumulación de la Ub^{G76V}- GFP en las células produciendo fluorescencia, lo cual permite una evaluación de la actividad proteolítica en los órganos y tejidos de interés. Sin

embargo, la fluorescencia directa que puede detectarse en el encéfalo de estos ratones tras la administración de fármacos inhibidores del proteasoma es baja (Lindsten *et al.*, 2003). Por ello, en la mayor parte de los estudios en los que se ha utilizado la línea Ub^{G76V}-GFP/1, se han empleado técnicas inmunohistoquímicas o de inmunofluorescencia para la detección de la GFP ligada a la ubiquitina (Kristiansen *et al.*, 2007; Ortega *et al.*, 2010; Quaglio *et al.*, 2011).

La utilización de esta línea transgénica ha permitido demostrar que parece existir una alteración del UPS en las áreas encefálicas con mayor acumulación de PrP^{Sc} en el estadio terminal de la enfermedad, lo cual se observó en ratones Ub^{G76V}-GFP/1 inoculados con la cepa 22L (Kristiansen *et al.*, 2007). Posteriormente, en ratones Ub^{G76V}-GFP/1 infectados con la cepa RML, también se observó que el deterioro del UPS en las neuronas y los astrocitos, en donde la Ub^{G76V}-GFP se acumulaba intensamente, parecía preceder a la aparición de los signos clínicos y que por tanto es un mecanismo patogénico temprano de las enfermedades priónicas (McKinnon *et al.*, 2016).

Finalmente, como se ha mencionado, el estrés prolongado del RE puede dar lugar a la activación de las caspasas y a la apoptosis neuronal, sin embargo, en las enfermedades priónicas, el papel de la apoptosis sigue siendo controvertido. En concreto en el scrapie natural, si bien sí se ha detectado la activación de vías apoptóticas (Serrano *et al.*, 2009) y sobreexpresión del factor pro-apoptótico Bax., no se ha detectado que exista una clara inducción de la apoptosis en la patogenia de esta enfermedad (Lyahyai *et al.*, 2006; Lyahyai *et al.*, 2007).

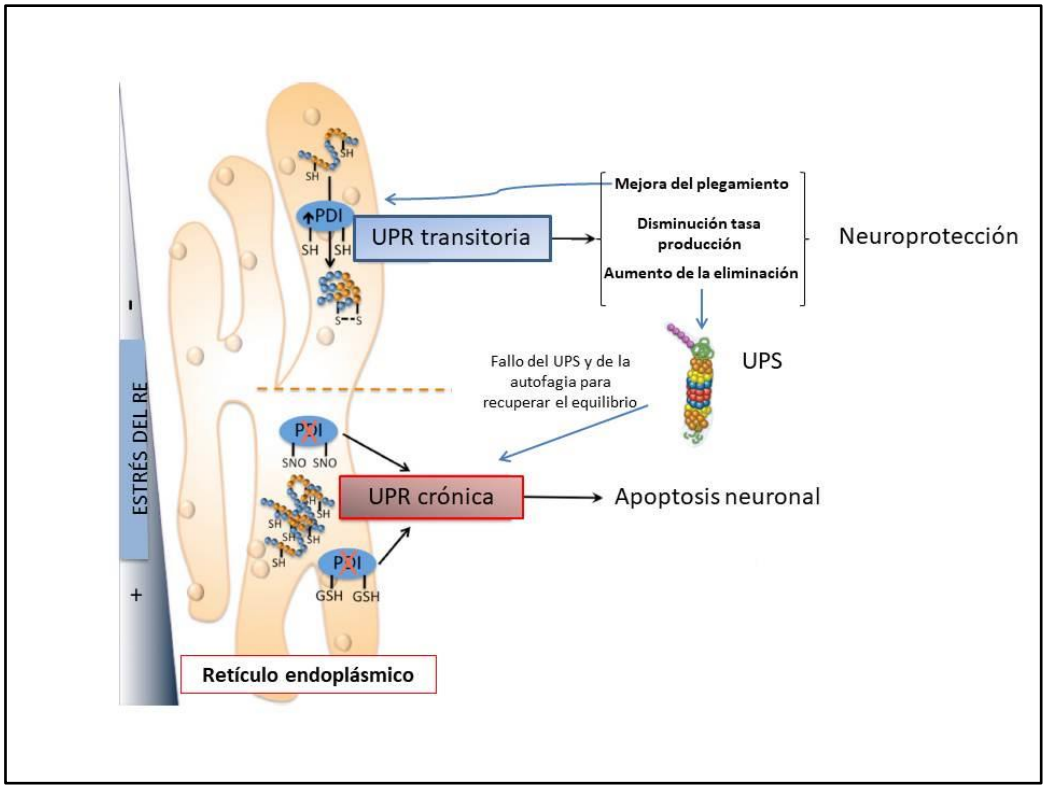

Figura 3: Representación esquemática de los mecanismos activados por el estrés del retículo endoplásmico (RE). Cuando se produce una acumulación de proteínas malplegadas en el interior del RE se produce una activación transitoria de la respuesta a proteínas malplegadas (UPR), que conduce a una mejora del plegamiento proteico mediante la sobreexpresión de ciertas proteínas, entre ellas la PDI. También se reduce temporalmente la producción de proteínas en el RE y aumenta la tasa de eliminación proteica, lo que se realiza a través del sistema ubiquitino-proteasómico (UPS), el mecanismo de eliminación de la vía de degradación asociada al RE. Cuando la acumulación de proteínas malplegadas y, por tanto, el estrés del RE persiste, se produce una S-nitrosilación de la PDI, haciendo que pierda su función. Esto, junto con el fracaso del UPS y de la autofagia para recuperar la proteostasis, conduce a una UPR crónica, lo cual finalmente lleva a la activación de las caspasas y a la muerte celular. Modificación de Grek and Townsend, 2014 y Ben-Nissan and Sharon, 2014.

ESTUDIO 1
An amino acid substitution found in animals with low susceptibility to prion diseases confers a protective dominant-negative effect in prion-infected transgenic mice

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An amino acid substitution found in animals with low susceptibility to prion diseases confers a protective dominant-negative effect in prion-infected transgenic mice

Abstract

While prion diseases have been described in numerous species, some, including those of the Canidae family, appear to show resistance or reduced susceptibility. A better understanding of the factors underlying prion susceptibility is crucial for the development of effective treatment and control measures. We recently demonstrated resistance to prion infection in mice overexpressing a mutated prion protein (PrP) carrying a specific amino acid substitution characteristic of canids. Here, we show that co-expression of this mutated PrP and wild-type mouse PrP in transgenic mice inoculated with different mouse-adapted prion strains (22L, ME7, RML, and 301C) significantly increases survival times (by 45% to 113%). These data indicate that this amino acid substitution confers a dominant-negative effect on PrP, attenuating the conversion of PrP^{C} to PrP^{Sc} and delaying disease onset without altering the neuropathological properties of the prion strains. Taken together, these findings have important implications for the development of new treatment approaches for prion diseases based on dominant-negative proteins.

Keywords

TSE; Prion infection; transgenic mouse models; transmissible spongiform encephalopathies; prion propagation; canine PrP

Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of neurodegenerative diseases of animals and humans that can be sporadic (putatively spontaneous), genetic, or acquired by infection (Prusiner, 1998a). TSEs are caused by the accumulation of a misfolded protein, the scrapie-associated prion protein (PrP^{Sc}), which is produced by posttranslational conversion of the physiologically expressed cellular prion protein (PrP^{C}) via an unknown mechanism. This abnormal form of the protein is protease resistant and is composed almost entirely of β-sheet structures (Prusiner, 1982, 1998b; Smirnovas *et al.*, 2011; Vazquez-Fernandez *et al.*, 2016). PrP^{Sc} deposition results in spongiosis, vacuolation, neuronal death, and glial reactions in the central nervous system of affected individuals (Fraser, 1976; Prusiner, 1982; Budka *et al.*, 1995; Vidal *et al.*, 2009)

TSEs naturally affect a wide variety of mammalian species, and include Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in cervids (Collins *et al.*, 2004). Since the emergence of BSE and its association with variant CJD (vCJD) in humans (Will *et al.*, 1996; Bruce *et al.*, 1997), transmission of prion diseases between species has become a major public health concern. Spongiform encephalopathies have been identified in numerous ruminant, feline, and primate species, all of which had consumed cattle meat or feed containing ruminant meat and bone meal, or were in close proximity to infected animals (Kirkwood *et al.*, 1993; Kirkwood and Cunningham, 1994; Sigurdson and Miller, 2003). However, the absence of prion diseases in other mammals exposed to contaminated food, including rabbits, equids, and canids, suggested the existence of prion-resistant species (Fernandez-Borges *et al.*, 2012). This was further supported by unsuccessful attempts to overcome TSE transmission barriers in those species, which contributed to preserve for decades the concept of prion resistant mammals (Fernandez-Borges *et al.*, 2009). Of all putative prion-resistant species, rabbits are the most extensively studied. *In vitro* studies using protein misfolding cyclic amplification (PMCA) and subsequent *in vivo* experiments have shown that rabbits are not disease-resistant *per se*, but are poorly susceptible to prion diseases (Chianini *et al.*, 2012). Equids represent a very interesting group of mammals that, while not completely resistant to TSEs, show a very peculiar susceptibility displaying an unusual replicative phenomenon termed nonadaptative prion amplification (NAPA), described in transgenic mice expressing horse PrP (Bian *et al.*, 2017). Moreover, the use of recombinant proteins

in the presence of chaotropic agents (Khan *et al.*, 2010) and *in vitro* prion amplification techniques (Vidal *et al.*, 2013) to study the propensity of prion protein misfolding in different mammalian species suggest that susceptibility to prion diseases is lowest in canids. To identify the specific features of canine PrP^{C} that account for its strong resistance to misfolding, we previously generated a transgenic mouse model expressing a PrP variant (N158D PrP), containing a single specific amino acid substitution, characteristic of the dog PrP^{C} (Fernandez-Borges *et al.*, 2017b). We found that this model was completely resistant to intracerebral infection with several mouse-adapted prion strains, indicating that a single amino acid substitution is sufficient to inhibit the misfolding of the mutated protein.

In the present study, we investigated whether this mutant could act as a dominant-negative protein and prevent PrP^{Sc} formation when co-expressed with wild-type (wt) PrP^{C}. To this end, we created a new mouse model co-expressing wt mouse PrP^{C} and the aforementioned mutant PrP variant carrying the critical dog amino acid substitution. These mice were intracerebrally inoculated with different mouse-adapted prion strains and the results of the *in vivo* challenge compared with those obtained in mice expressing comparable levels of wt mouse prion protein. Surprisingly, co-expression of the mutated protein significantly delayed the onset of disease induced by all prion strains studied. Survival periods were increased by 45% to 113% with respect to mice expressing wt protein alone, thereby demonstrating the dominant-negative effect of the mutant protein. Our findings show that this specific dog amino acid substitution confers the protein the ability to interfere with the propagation of wt prions in transgenic mice. These findings have important implications for the development of therapeutic strategies against prion diseases.

Materials and methods

Generation and inoculation of transgenic mouse models

Three different transgenic mouse models were used in the present study: 1) Tga20 x Tga20 mice (hereafter referred to as Tga20 mice) expressing mouse PrP^{C} at a levels ~8-fold higher than those observed in the mouse brain (Fischer *et al.*, 1996); Tga20 x $Prnp^{0/0}$ (Manson *et al.*, 1994) (hereafter referred to as Tga20xKO mice) mice expressing mouse PrP^{C} at a levels ~4-fold higher than those observed in mouse brain; and 3) Tga20 x TgN158D mice [hereafter referred to as Tga20xN158D (Fernandez-Borges *et al.*, 2017b) expressing mouse PrP^{C} at levels ~4-fold higher and N158D mouse PrP^{C} at levels

~2-fold higher than those observed in mouse brain. The murine *PRNP* promoter was used for both wt and N158D mouse PrP^C.

PrP expression levels from Tga20 x Tga20, Tga20xKO and Tga20xN158D mice were analyzed by Western blot using SAF83 (1:400) and 5C6 (1:2000) monoclonal antibodies and compared with those obtained in TgN158DxTgN158D mice (hereafter referred to as TgN158D mice), expressing only N158D mouse PrP^C (Fernandez-Borges *et al.*, 2017b). 5C6 antibody (PRC5 antibody) was kindly provided by Dr. Glenn Telling (Prion Research Center, Colorado State University). This antibody requires asparagine at mouse PrP residue 158 (Kang *et al.*, 2012) and therefore does not detect N158D PrP, whereas SAF83 antibody recognizes both wt and N158D mouse PrPs. Western blot results reflect the aforementioned wt and N158D PrP expression levels. No differences were observed regarding the PrP electrophoretic migration patterns between the different groups of mice (Supplementary Fig. 1).

Mice aged 6 to 8 weeks were anesthetized with isoflurane and intracerebrally inoculated (left cerebral hemisphere) with mouse-adapted prion strains 22L, RML, ME7, or 301C using 20 μl of a 10% brain homogenate. Injections were administered using a 50-μl syringe and a 25-G needle. Analgesia was achieved by subcutaneous injection of buprenorphine (0.3 mg/kg). Animals were subsequently housed in filtered cages and monitored three times per week for neurologic dysfunction. Mice were euthanized by cervical dislocation upon detection of clinical signs of terminal disease (severe ataxia, inability to stand, and poor body condition).

All experimental procedures were approved by the Ethics Committee for Animal Experiments of the University of Zaragoza (permit number: PI32/13) and performed in accordance with the recommendations for the care and use of experimental animals and in agreement with Spanish law (R.D. 1201/05).

Sample processing and histopathological evaluation

After euthanasia, brains were removed, and transversal sections from the frontal cortex and medulla oblongata were separated and frozen at -80°C for subsequent biochemical analyses. The remaining tissue was fixed in 10% formalin for neuropathological studies. After fixation, brains were cut at four standard levels for the histological evaluation of the following 9 brain regions: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellum (Cbl) and medulla oblongata (Mo) (Fraser and

Dickinson, 1968). Tissues were embedded in paraffin, cut into 4-µm-thick sections on a microtome, and mounted on glass slides for staining with hematoxylin and eosin. Sections were examined using an optical microscope (Zeiss Axioskop 40), and the extent of vacuolation and spongiosis in each area was blindly evaluated and semi-quantitatively scored on a scale of 0 (absence of lesions) to 5 (high intensity lesions).

Analysis of PrP^{Sc} deposition

The intensity and distribution of PrP^{Sc} deposition was evaluated using the paraffin-embedded tissue (PET) blot method, as previously described (Schulz-Schaeffer *et al.*, 2000). Sections from paraffin-embedded brains (4-µm thick) were collected on a nitrocellulose membrane (0.45-µm pore size; Bio-Rad, Richmond, CA) and dried at 55°C for 24 h. After deparaffination and rehydration, sections were digested for 2 h at 56°C with 250 µg/ml proteinase-K (PK) (Applied Biosystems) in PK digestion buffer containing TBS (Tris-buffered saline) and 0.1% Brij 35P (Sigma-Aldrich). After washing with TBST (Tris buffered saline; 0.05% Tween 20), membrane-attached proteins were denatured in 3 M guanidine thiocyanate (Sigma-Aldrich). Sections were then blocked with 1% casein in TBST and incubated with Sha31 primary monoclonal antibody (1:8000; SPI-Bio). After incubation with an alkaline phosphatase-coupled goat anti-mouse antibody (DAKO) immunostaining was visualized using NBT/BCIP (Nitro blue tetrazolium /5-bromo-4-chloro-3-indolyl-phosphate; Sigma-Aldich). PrP^{Sc} deposits were evaluated semi-quantitatively, as described for spongiform lesions, using a Zeiss Stemi DV4 stereo microscope.

Histological analysis of PrP^{C} distribution

The localization and distribution of PrP^{C} in the brains of Tga20xN158D mice was analyzed by immunohistochemistry. Brains from TgN158D mice were used as controls. Serial paraffin-embedded sections were incubated with a peroxidase blocking reagent (Dako) for 20 min followed by hidrated autoclaving at 100°C in citrate buffer for 30 min. Immunodetection was performed overnight at 4°C using SAF32 (1:1000; SPI-Bio) and 5C6 (1:1000) anti-PrP monoclonal antibodies. The anti-mouse Envision polymer (Dako) was used as the visualization system and DAB (diaminobenzidine, Dako) as the chromogen.

The localization of N158D PrP was analyzed using immunofluorescence and confocal imaging. Immunofluorescence staining was performed as described previously

(Sarasa *et al.*, 2012), with specific modifications to adapt the protocol to paraffin-embedded samples. Paraffin-embedded tissue sections from TgN158D mice were deparaffinated an rehydraded and then blocked with 1% H2O2 for 30 min. After a pretreatment with 0,1% Triton X-100 for 3 h at room temperature, samples were subjected to hidrated autoclaving and incubated with SAF32 antibody (1:100) followed by a goat anti-mouse IgG biotin conjugate (1:100; Invitrogen) and a Alexa fluor 594 streptavidin conjugate (1:1000; Invitrogen). Sections were analyzed using a Zeiss laser-scanning confocal microscope LSM 510 (Carl Zeiss MicroImaging).

Biochemical analysis

Frozen brain sections stored for biochemical analysis, as described above, were homogenized in 1% (w/v) in PBS (phosphate buffered saline) using a ribolyzer. The resulting samples were digested with Protease K (PK) for 1 h at 42°C and used for Western blot. Immunodetection was performed using SAF83 (SPI-Bio; 1:400) and 5C6 (1:2000) primary antibodies.

Data analysis

Survival times were analyzed by Kaplan-Meier survival analysis, and the resulting survival curves were compared using the log rank test (α=0.050). Differences in spongiform lesions (distribution and intensity) and PrP^{Sc} deposition profiles between different transgenic mouse models were evaluated using the non-parametric Mann-Whitney *U*-test and considered significant at $p < 0.05$. GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA) was used to perform all statistical analyses, generate Kaplan Meier curves, and to graph histopathology results.

Results

Co-expression of the N158D PrP substitution greatly increases survival time in inoculated mice

Three groups of mice expressing different levels of wt protein, either alone or together with N158D PrP [(Tga20 (8x), Tga20xKO (4x+0x) and Tga20xN158D (4x+2x)] (Supplementary Fig. 1) were challenged by intracerebral inoculation with the 22L, RML, 301C, or ME7 mouse-adapted prion strains. Owing to its high levels of PrP^{C} expression in the brain (~8 fold higher than wt mice), the TSE incubation period in the Tga20 mouse is relatively short, making it a useful model for prion research. Moreover, the histopathological and biochemical features of several mouse-adapted TSE strains,

including those used in the present study, are well defined in this transgenic model (Fischer *et al.*, 1996; Karapetyan *et al.*, 2009). Tga20xN158D mice were used to achieve co-expression of the wt and N158D PrP. Tga20xKO mice were selected as controls, given that their wt PrP^C expression level is identical to that of Tga20xN158D mice. No significant differences were appreciated in electrophoretic migration patterns between the different mice lines (Supplementary Fig. 1). In addition, both wt and N158D PrPs are present in high amounts and are normally distributed through the brain in transgenic mice (Supplementary Fig. 2).

Tga20xN158D mice inoculated with the 22L, RML, 301C, or ME7 prion strains showed increases in survival times of 113%, 45%, 71%, and 49%, respectively, as compared with Tga20xKO mice, which express the same amount of wt PrP^C (Table 1). Significant differences were observed between genotypes for all inocula (Fig. 1). In the case of the 301C strain, survival times in Tga20xN158D mice were not as homogeneous as those observed for the other strains, as evidenced by less steep decline in the Kaplan-Meier curve (Fig. 1).

Although survival time was significantly increased in Tga20xN158D mice, the clinical presentation in these mice was indistinguishable from that of mice expressing only the wt protein. Mice developed clinical signs typical of TSEs in rodents, including poor hair coat condition and kyphosis in the early stages of the disease, followed by proprioceptive deficits, head twitching, and progressive ataxia, which became severe in terminal stages. However, certain clinical signs were more evident in animals infected with a given inoculum (e.g., the opisthotonos observed in all three genotypes of mice inoculated with the 22L strain, a sign that may be due to cerebellar lesions).

Table 1 Inoculation of Tga20, Tga20xKO, and Tga20xN158D mice with mouse-adapted prion strains

PrP expression levels						
Inoculum	Model	wt	Mutant (N158D)	Attack rate[a]	Survival time (dpi) (mean ± SEM)[b]	Relative increase in survival time (%)[c]
22L	Tga20xTga20	8×	0×	6/6 (100%)	91 ± 2	–
	Tga20xKO	4×	0×	6/6 (100%)	98 ± 2	–
	Tga20xN158D	4×	2×	11[d]/11 (100%)	209 ± 3	113%
RML	Tga20xTga20	8×	0×	6/6 (100%)	70 ± 3	–
	Tga20xKO	4×	0×	6/6 (100%)	88 ± 1	–
	Tga20xN158D	4×	2×	11[d]/11 (100%)	128 ± 3	45%
301C	Tga20xTga20	8×	0×	6/6 (100%)	75 ± 1	–
	Tga20xKO	4×	0×	6/6 (100%)	92 ± 4	–
	Tga20xN158D	4×	2×	12/12 (100%)	157 ± 17	71%
ME7	Tga20xTga20	8×	0×	6/6 (100%)	96 ± 2	–
	Tga20xKO	4×	0×	6/6 (100%)	101 ± 2	–
	Tga20xN158D	4×	2×	10[d] /10 (100%)	150 ± 3	49%

[a] Data based on PrP^{res} detection

[b] Survival times were calculated as the number of days between inoculation and euthanasia, provided that the mouse developed clinical signs consistent with a TSE. Survival times are expressed as mean (± SEM) number of dpi

SEM standard error of the mean, *dpi* days postinoculation

[c] Extension of the survival times in Tga20xN158D mice inoculated with each strain was calculated as the difference between the average survival time of Tga20xN158D and that of Tga20xKO expressed in relative percentages to the average survival times of Tga20xKO

[d] Animals from the 22L (1), RML (1), and ME7 (2) inoculation groups died due to concomitant diseases during the initial stages of the study and were excluded from the analyses. These animals exhibited no spongiform lesions or PrP^{Sc} deposits and were not included in calculations of the SEM or attack rate

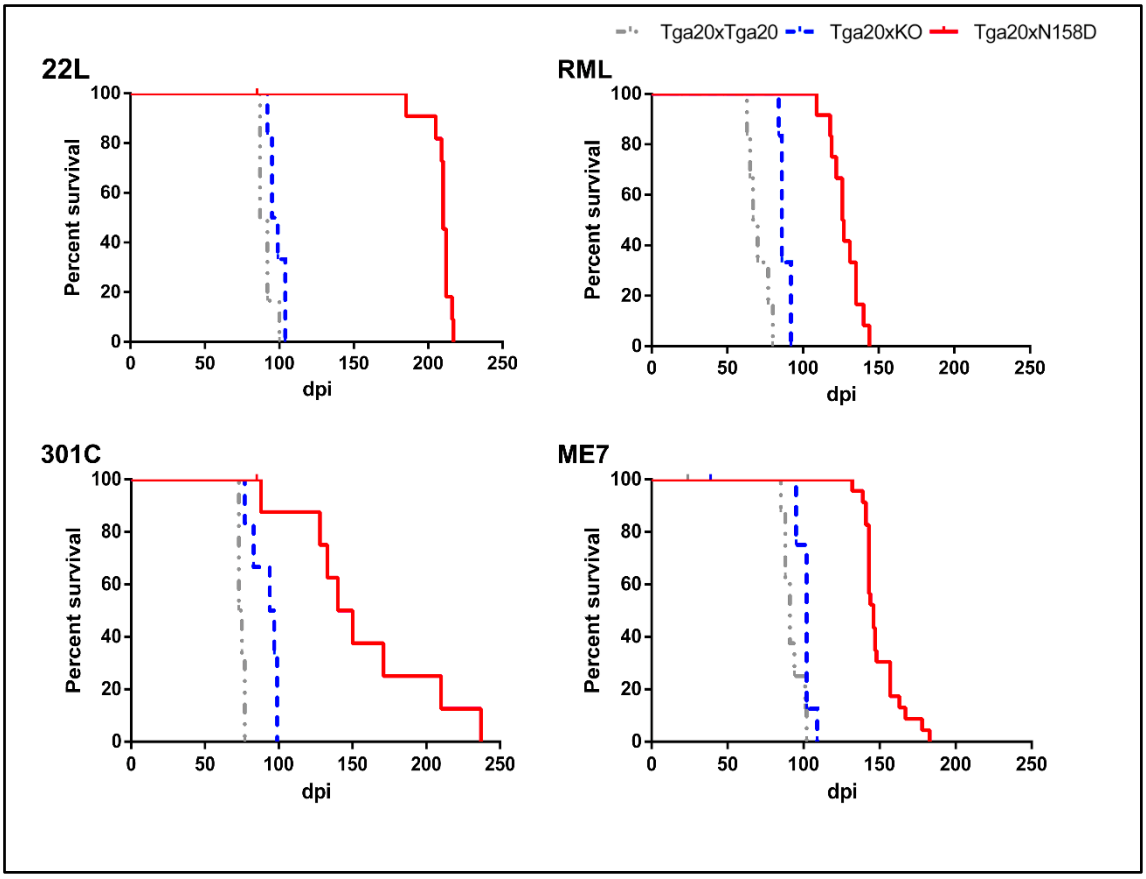

Figure. 1: Survival curves for Tga20, Tga20xKO, and Tga20xN158D mice challenged with different mouse-adapted prion strains. Comparison of Tga20xN158D curves with Tga20xKO curves using the log rank test (α=0.050) revealed very significant differences for the 22L, RML, and ME7 ($p<0.0001$) and the 301C ($p<0.0033$) inoculation groups. Survival curves for Tga20 mice inoculated with the corresponding strains are also shown. Tga20xN158D mice infected with the 22L, RML, 301C, or ME7 prion strains showed relative increases in survival times of 113%, 45%, 71%, and 49%, respectively when compared with those of Tga20xKO mice.

Expression of the dominant-negative protein did not alter the neuropathological features of the disease

Despite the substantial prolongation of survival time in mice co-expressing the N158D PrP substitution, an exhaustive comparison of the two models expressing equivalent levels of wt PrP^C (Tga20xKO and Tga20xN158D mice) revealed no significant differences in terms of the neuropathological characteristics of the disease. Lesion profiles and prion protein deposition patterns, evaluated semi-quantitatively on a scale of 0 to 5, were very similar between different genotypes inoculated with the same strain (Figs. 2 and 3). Our results are consistent with those of another study in which Tga20 mice were infected with all the same inocula (Karapetyan *et al.*, 2009), indicating that the

mouse-adapted strains used in the present study retained their characteristic histopathological features and PrP^{Sc} deposition profiles.

All mice infected with the 22L strain developed particularly severe spongiform lesions and showed marked PrP^{Sc} deposition in the T, Ht, Mes and Mo (Figs. 2 and 3a). Inoculation with the RML strain resulted in intense histopathological changes and PrP^{Sc} deposition predominantly in the T, Mes, and Mo (Figs. 2 and 3a), with low vacuolation scores observed in the Cbl. The spongiform lesions caused by the 301C strain were mainly located in the T, Mes, and Mo (Fig. 2). Compared with the other strains used, this mouse-adapted BSE strain produced slightly less intense PrP^{Sc} deposition throughout the brain (Fig. 3a), as described previously in wt mice (Tuzi *et al.*, 2008). Finally, inoculation with ME7 resulted in severe spongiosis and vacuolation in the Sa, T, Ht, and brainstem (Fig. 2) and very intense PrP^{Sc} deposition in the Hc, T, and Sa in all genotypes, (Fig. 3a), indicating that the main features of the ME7 strain, as previously described in Tga20 mice (Karapetyan *et al.*, 2009), were preserved (Fig. 3b).

The results of biochemical analyses were consistent with the histopathological findings. No significant differences in PrP glycosylation patterns between Tga20, Tga20xKO, and Tga20xN158D mice were observed for any of the strains inoculated (Fig.4). We further investigated whether the similarities observed between Tga20xKO and Tga20xN158D mice regarding the histopathological and biochemical features of the disease could be related to an exclusive conversion of wt PrP^{C}. Serial dilutions of brain homogenates from 22L infected Tga20xKO and Tga20xN158D mice were analyzed for PrP^{res} using two different antibodies: 5C6, which is unable to detect N158D PrP since it requires the presence of asparagine at codon 158 (Kang *et al.*, 2012), and SAF83, which detects both wt and N158D PrPs. No differences were observed in the amount of PrP^{res} detected by these antibodies in Tga20xN158D mice, suggesting that only wt PrP^{C} was converted (Supplementary Fig. 3).

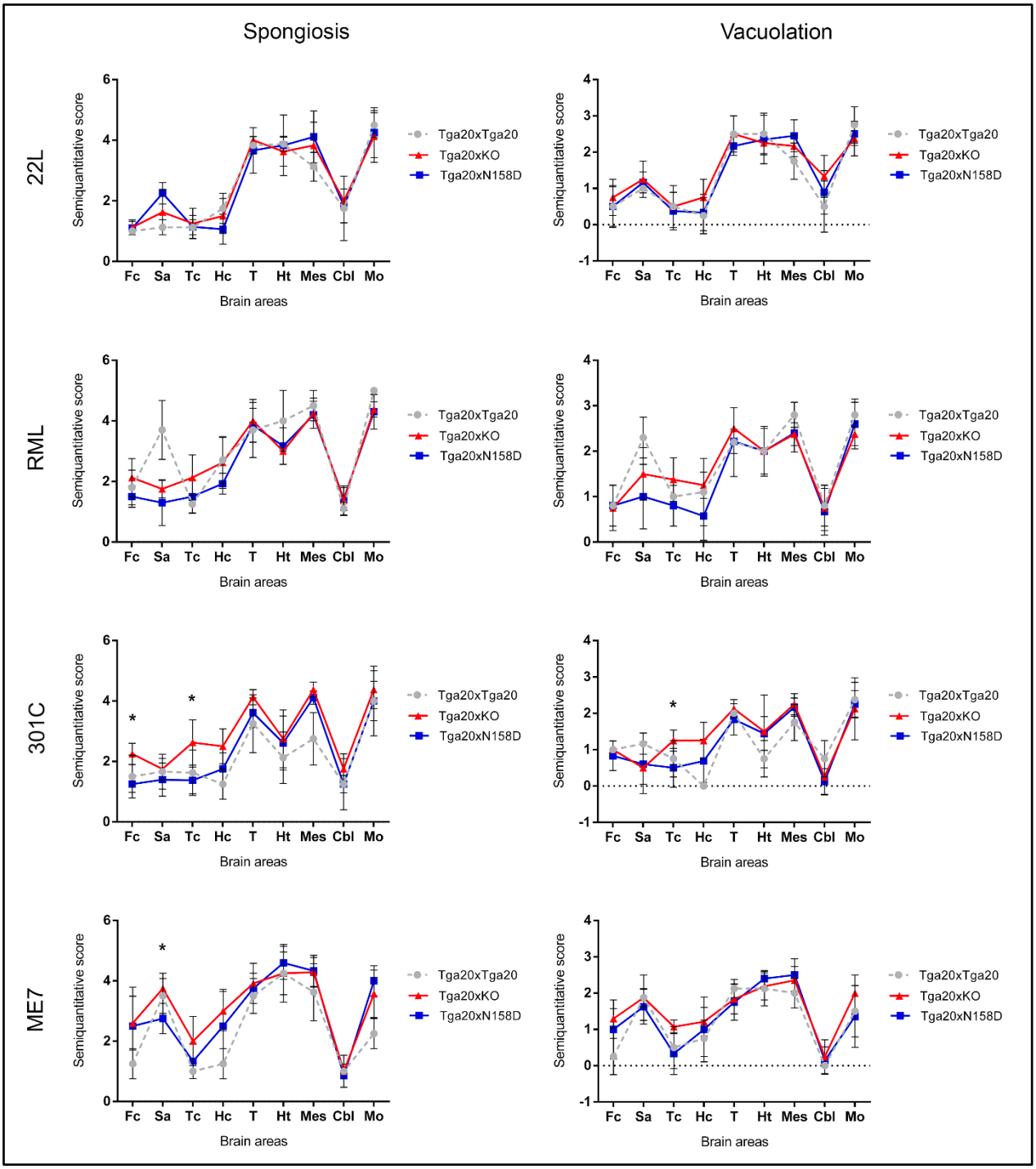

Figure 2: Brain lesion profiles of Tga20, Tga20xKO, and Tga20xN158D mice inoculated with different mouse-adapted prion strains. Spongiosis and vacuolation were evaluated semiquantitatively on a scale of 0 (absence of lesions) to 5 (high intensity lesions) in the following 9 brain areas: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellum (Cbl) and medulla oblongata (Mo). Comparison

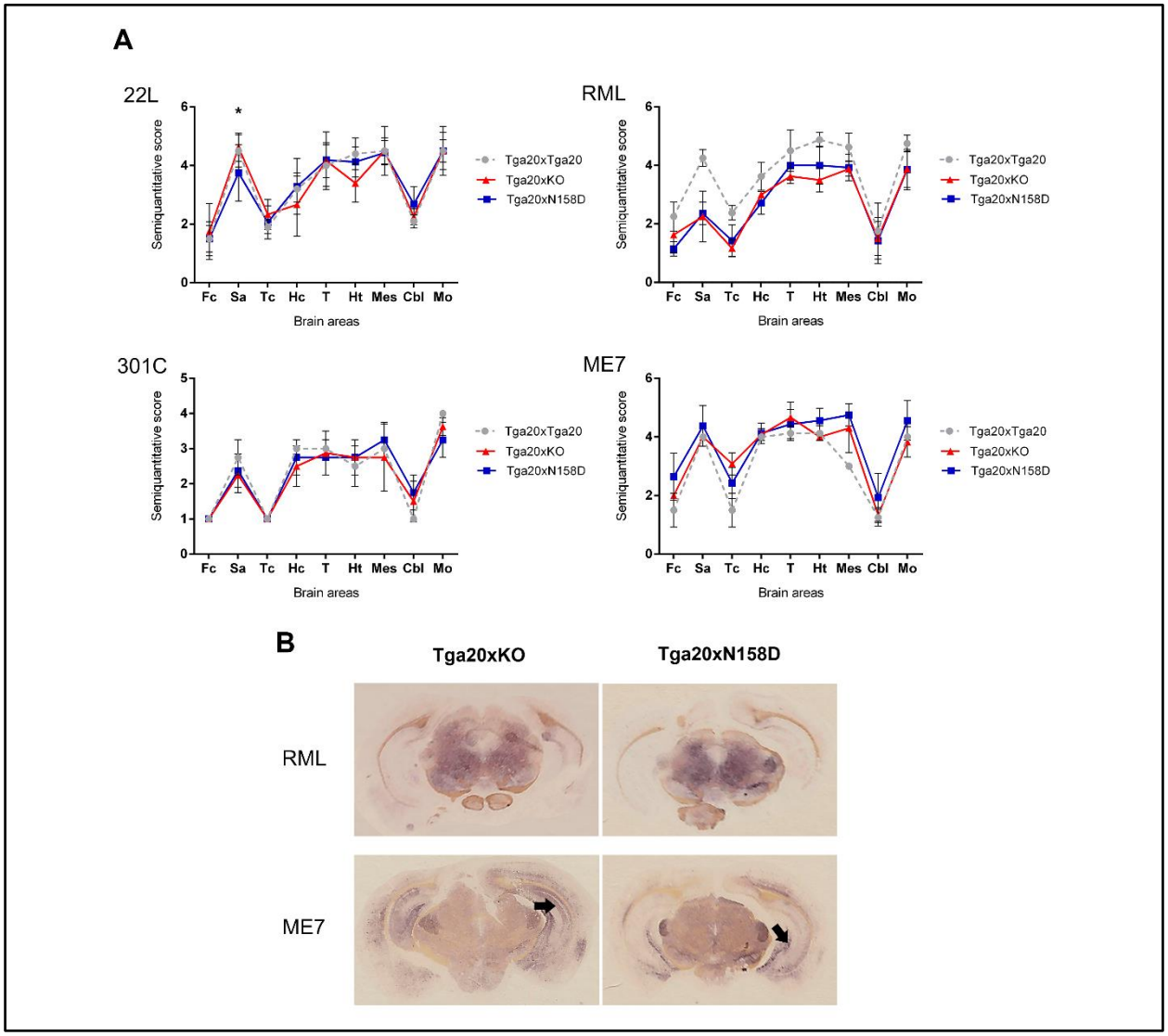

Figure 3: (a) PrP^{Sc} deposition profiles in the brains of Tga20, Tga20xKO, and Tga20xN158D mice inoculated with 22L, RML, 301C, or ME7 prion strains. PrP^{Sc} deposition was evaluated semiquantitatively on a scale of 0 (absence of deposits) to 5 (high intensity deposition) in the following 9 brain areas: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellum (Cbl) and medulla oblongata (Mo). Comparison of the PrP^{Sc} deposition profiles of Tga20xKO and Tga20xN158D mice revealed almost identical PrP^{Sc} profiles (*$p<0.05$, Mann-Whitney *U*-test). **(b)** PET blot images of coronal sections of the mesencephalon from Tga20xKO and Tga20xN158D mice inoculated with the RML or ME7 strain. Note that the PrP^{Sc} deposition profile of mice expressing the mutant PrP is almost identical to that of Tga20xKO mice. Moreover, the characteristic deposition patterns of the inoculated strains are retained: note the marked deposition in the hippocampus in ME7-inoculated mice (arrows), a feature not observed in RML-inoculated mice.

Discussion

Certain PrP polymorphisms are strongly linked to susceptibility/resistance to prion diseases. This relationship has been well documented in sheep, leading to the establishment of five haplotypic PrP gene variants associated with scrapie susceptibility (Belt *et al.*, 1995). Among the three main polymorphisms of ovine *PRNP*, variations at codon 171 appear to be the principal determinants of resistance to classical scrapie; sheep with arginine at this specific residue are resistant to natural (Westaway *et al.*, 1994b) and experimental (Clouscard *et al.*, 1995; Hunter *et al.*, 1997) scrapie infection. Heterozygosity at certain PrP positions also exerts protective effects against human prion diseases (Shibuya *et al.*, 1998).

The ability of certain variant proteins to interfere with co-expressed wt PrP and block prion replication is known as a dominant-negative effect. This has been experimentally reproduced in cells and in transgenic mice, and may have implications for the development of therapeutic strategies for prion diseases (Kaneko *et al.*, 1997b; Zulianello *et al.*, 2000; Perrier *et al.*, 2002; Lee *et al.*, 2007). The use of PMCA in *in vitro* studies has proved an efficient means of testing a wide variety of PrPs with different substitutions in order to identify the most appropriate dominant-negative changes (Fernandez-Borges *et al.*, 2009).

In the search for PrPs that exert a consistent and potent inhibitory effect on *in vivo* prion propagation, it seems reasonable to begin with PrPs from species with demonstrated low susceptibility to prion diseases. For the purposes of this study, we selected dog prion protein, in which low susceptibility has been proven (Khan *et al.*, 2010; Vidal *et al.*, 2013). Using cell and brain-based PMCA, we previously demonstrated that the substitution of asparagine with aspartic or glutamic acid at codon 163, a distinctive substitution from the Canidae family (Stewart *et al.*, 2012), strongly inhibits prion replication *in vitro*. Moreover, we found that when transgenic mice overexpressing a PrP variant carrying this substitution were challenged with several mouse-adapted prion strains, they were completely resistant to prion infection (Fernandez-Borges *et al.*, 2017b). Based on these findings, we investigated whether the co-expression of this mutant PrP together with wt mouse PrP could interfere with prion propagation, thereby preventing or delaying the onset of the disease *in vivo*.

Co-expression of both proteins dramatically increased survival times after inoculation with any of the four mouse-adapted prion strains tested (22L, RML, 301C and

ME7). Furthermore, survival times in Tga20 and Tga20xKO mice differed significantly. This was not unexpected since PrP^C expression levels dramatically influence the incubation time in prion diseases, and expression levels of PrP^C are inversely proportional to the duration of the survival period (Prusiner *et al.*, 1990; Bueler *et al.*, 1994). However, it is important to note that, in our study, the appropriate comparison of survival period is with that of mice expressing an equivalent amount of wt PrP (i.e., Tga20xKO vs. Tga20xN158D mice; Table 1).

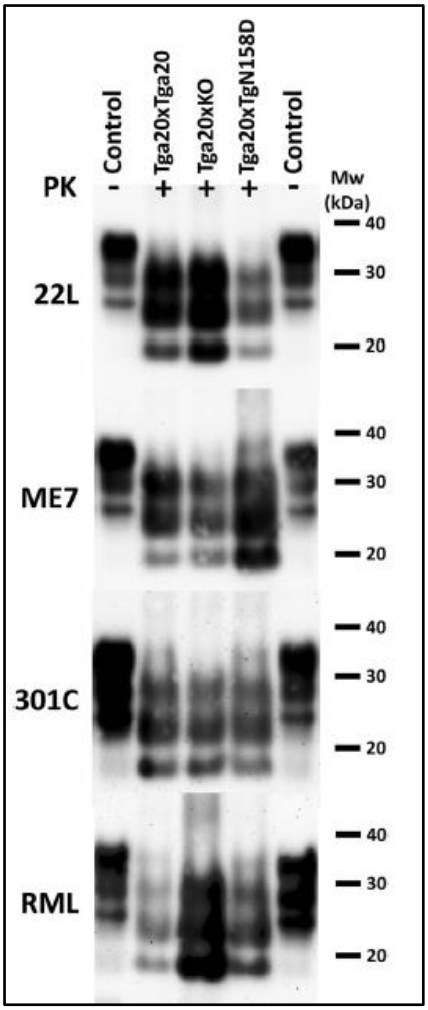

Figure 4: PrP^{res} detection from 22L, ME7, 301C, and RML inoculated Tga20, Tga20xKO, and Tga20xN158D mouse brains. Ten percent brain homogenates from 22L, ME7, 301C, and RML inoculated Tga20, Tga20xKO, and Tga20xN158D mice were digested with 80 µg/ml of Protease K (PK). Digested samples were analyzed by Western blot using SAF83 (1:400). No significant differences are observed between any of the Tga20, Tga20xKO, and Tga20xN158D brain homogenates suggesting that N158D PrP^C did not alter the major biochemical characteristics of any of the four prion strains. Control: undigested Tga20xKO brain homogenate. Mw molecular weight

The elongation of the survival times produced by the co-expression of an exogenous protein can be the result of several processes. We observed that, when PrP^{res} levels from Tga20xKO and Tga20xN158D mice culled at different days post inoculation (dpi) were compared, Tga20xKO mice showed higher amounts of PrP^{res}, even at shorter incubation periods than Tga20xN158D mice (Supplementary Fig. 4). Thus, we can suggest that the longer survival times observed in Tga20xN158D mice may be due to a

slower rate of misfolding of the wt PrP, therefore producing a more retarded accumulation of PrP^{Sc}. However, the molecular mechanisms by which N158D PrP delays prion propagation remain unclear. Several theories, most of them developed using scrapie infected cell models, have been proposed to explain how dominant-negative determinants inhibit prion propagation. It has been suggested that dominant-negative proteins, although differing only at one position from the wt PrP, obstruct the interactions between similar PrP monomers (Hope *et al.*, 1986; Bolton and Bendheim, 1988; Prusiner *et al.*, 1990; Priola *et al.*, 1994) and that the differences between mutant and wt PrP make both proteins structurally incompatible (Jahandideh *et al.*, 2015). This dissimilarity could interfere with the rate of formation (Lee *et al.*, 2007) and the stability of PrP^{Sc} polymers (Priola *et al.*, 1994; Geoghegan *et al.*, 2009). In addition, it has been also proposed that dominant-negative proteins may compete with wt PrP^{C} for binding to newly formed PrP^{Sc} molecules (Geoghegan *et al.*, 2009; Yuan *et al.*, 2013). Thus, the prolongation in survival times observed in the present study might also be the result of a greater affinity of N158D PrP for interacting with PrP^{Sc} than that of wt PrP. Due to the apparent resistance of N158D PrP to misfold (Supplementary Fig. 3), a competition of this mutant and wt PrP for the same binding site in PrP^{Sc} would explain the delay of the disease observed in Tga20xN158D mice, as previously reported (Geoghegan *et al.*, 2009; Yuan *et al.*, 2013).

Although survival times were significantly increased in Tga20xN158D mice inoculated with all experimental strains, this effect was not homogeneous for all strains. The greatest increase was observed in mice inoculated with the 22L strain: survival time in mice carrying the N158D PrP variant was 113% longer than that of controls. The smallest increase in survival times was observed in RML-inoculated Tga20xN158D mice (45% increase). It is well demonstrated that when propagated *in vivo,* distinct mouse-adapted prion strains differ in terms of incubation period, as well as their biochemical and neuropathological features (Fraser and Dickinson, 1973; Bruce *et al.*, 1991; Bruce, 1993; Baron *et al.*, 2004). Strains can also show biophysical, molecular, and, as in the case of the strains used in the present study, ultrastructural differences (Kascsak *et al.*, 1985; Sim and Caughey, 2009). These findings could explain that different tertiary and/or quaternary structures were also differentially affected by the blockade of a dominant-negative protein. Our findings suggest that the dominant-negative effect of this mutant protein is stronger with certain strains (22L and 301C) than with others (RML and ME7). Other dominant-negative proteins have been also reported to interfere with the generation of

PrP^{Sc} in a strain-specific manner. As an example, Q218K PrP strongly inhibits the misfolding of co-expressed wt PrP in Chandler-infected cells but produces a much weaker inhibition with 22L strain. This distinct effect was attributed to the structural differences, determined by IR spectroscopy, between Chandler and 22L strains (Atarashi *et al.*, 2006). As aforementioned, we cannot know for certain what precise molecular mechanisms are involved in the partial dominance exerted by N158D PrP. However, if the dominant-negative protein blocks fibril growth, ultrastructural differences between strains could account for the differential effect (45%–113% increase) of the dominant-negative protein on the survival period.

The dominant-negative effect of certain mutant PrPs on PrP^{Sc} formation has been already demonstrated *in vivo* in transgenic mice co-expressing wt PrP (Perrier *et al.*, 2002). In that study, mice expressing PrPs containing ovine and human TSE resistance-associated substitutions were not completely resistant to prion formation when mutant and wt mouse PrPs were co-expressed. Our findings are in agreement with those results and demonstrate that minimal amino acid changes can produce highly efficient dominant-negative variants able to double the survival period when co-expressed with wt mouse PrP^{C}. Extrapolating these findings to humans, in which the incubation period of prion diseases can last for decades, it seems possible that affected individuals may never develop clinical signs. The ability shown by certain additional PrP molecules with single residue substitutions to interfere with the misfolding of the endogenous PrP^{C}, has already been demonstrated. However, most of the approaches have been performed using cell cultures (Priola *et al.*, 1994; Kaneko *et al.*, 1997b; Atarashi *et al.*, 2006), whereas *in vivo* studies are limited (Telling *et al.*, 1995; Perrier *et al.*, 2002). In addition, most of the dominant-negative effects described for this type of molecules have been, albeit potent, demonstrated against a limited number of strains (Priola *et al.*, 1994; Perrier *et al.*, 2002; Atarashi *et al.*, 2006). We have shown that N158D PrP produces a dominant-negative inhibiton in the prion propagation of a variety of strains, of both scrapie (22L, RML and ME7) and BSE (301C) origins. The delay of the disease was not homogeneous among the strains despite showing inhibition against the propagation of all of them. When describing dominant-negative PrPs, it is important to check their ability to interfere with the propagation of prions from different origins and characteristics. Other naturally occurring amino acid variants of PrP^{C}, such as sheep Q171R, have demonstrated a strong dominant-negative inhibition in the propagation of scrapie strains (Kaneko *et al.*, 1997b; Zulianello *et al.*, 2000; Perrier *et al.*, 2002; Geoghegan *et al.*, 2009). However, it has been

shown that sheep with Q171R are susceptible to atypical scrapie (Buschmann *et al.*, 2004) as well as BSE (Houston *et al.*, 2003). Thus, our study suggests that N158D PrP, a substitution found in canids, in which no natural prion diseases have been reported, may be a dominant-negative protein with a broader inhibitory effect.

The prolongation of the incubation period seen in the present study was less dramatic than that reported by Perrier and coworkers in RML-inoculated mice co-expressing equal amounts of wt PrP and dominant-negative PrP. However, wt PrP expression levels in our Tga20xN158D mice are four times higher than those of wt mice, making it more difficult to fully block prion formation. It cannot be ruled out that if an equimolecular amount of dominant-negative PrP and wt PrP is required for the complete blockade of prion propagation, we would need to double the amount of N158D PrP. The dose dependent, dominant-negative inhibition by other similar molecules has already been demonstrated (Priola *et al.*, 1994; Perrier *et al.*, 2002; Geoghegan *et al.*, 2009), showing that certain dominant-negative proteins need to be present in high amounts to inhibit endogenous PrP^{C} conversion (Perrier *et al.*, 2002; Geoghegan *et al.*, 2009). We have observed that N158D PrP, even being expressed at lower levels than wt PrP, is able to significantly extend the survival period in Tga20xN158D mice.

The neuropathological changes seen in our Tga20xN158D mice were very similar to those observed in mice expressing only wt PrP, with few significant differences observed in terms of lesion and PrP^{Sc} deposition profiles (Figs. 2 and 3). These findings, coupled with the complete resistance to intracerebral challenge seen in mice expressing N158D mutant protein only (Fernandez-Borges *et al.*, 2017b), could lead us to think that the pathological form detected, and therefore, the neuropathological hallmarks observed in Tga20xN158D mice are due only to the conversion of the mouse wt protein. Fortunately, the expression of aspartic acid at 158 residue of mouse N158D PrP^{C} impedes the epitope recognition of 5C6 antibody (Kang *et al.*, 2012), and therefore it allows discrimination between wt and N158D PrP^{C} .

Our results indicate that only mouse wt PrP^{C} was converted in Tga20xN158D mice (Supplementary Fig. 3). Accordingly with this suggestion, most of the pathological features previously described in Tga20 mice inoculated with the strains used in the present study (Karapetyan *et al.*, 2009) were reproduced in Tga20xN158D mice. All of the prion strains tested produced marked spongiosis and PrP^{Sc} deposition in both the thalamus and brainstem of Tga20xN158D mice (Figs. 2 and 3), regions previously proposed as clinical

target areas of these strains in Tga20 mice (Karapetyan *et al.*, 2009). In mice co-expressing N158D PrP, these different prion strains retained their specific pathological characteristics, as evidenced by the marked PrP^{Sc} deposition in the hippocampus of ME7-inoculated mice (Fig. 3b) (Karapetyan *et al.*, 2009) and the characteristic affectation of the cerebellum in those inoculated with the 22L strain (Fraser, 1979). Expression of the dominant-negative protein therefore appears not to have affected the characteristic pathological hallmarks of these strains, indicating that the increase in survival times observed in Tga20xN158D mice is not due to strain modifications caused by the amino acid substitution of the dominant-negative protein.

Based on our findings, we conclude that N158D PrP acts as a dominant-negative protein to partially block the conversion of PrP^{C} to PrP^{Sc} and is thus a promising candidate for gene therapy strategies for the treatment of TSEs.

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Compliance with ethical standards

This study was approved by the Ethics Committee for Animal Experiments of the University of Zaragoza (permit number PI32/13) and was performed in accordance with the recommendations for the care and use of experimental animals and with Spanish national law (R.D. 1201/05).

Conflict of interest

The authors declare that they have no conflict of interest.

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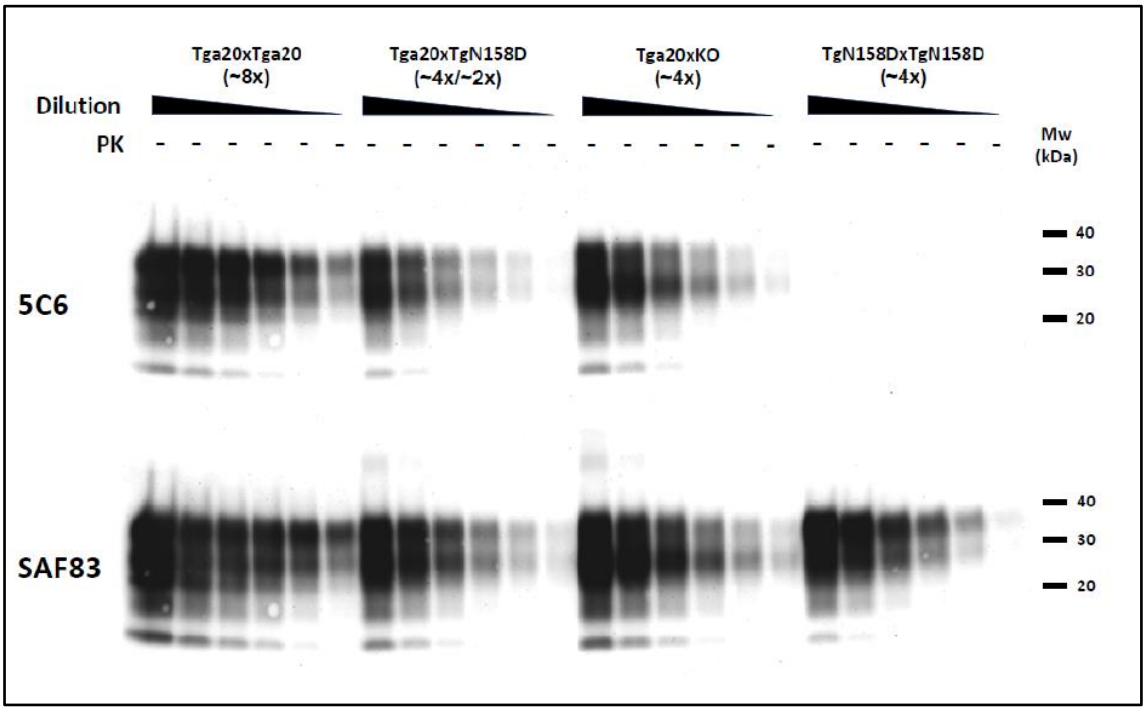
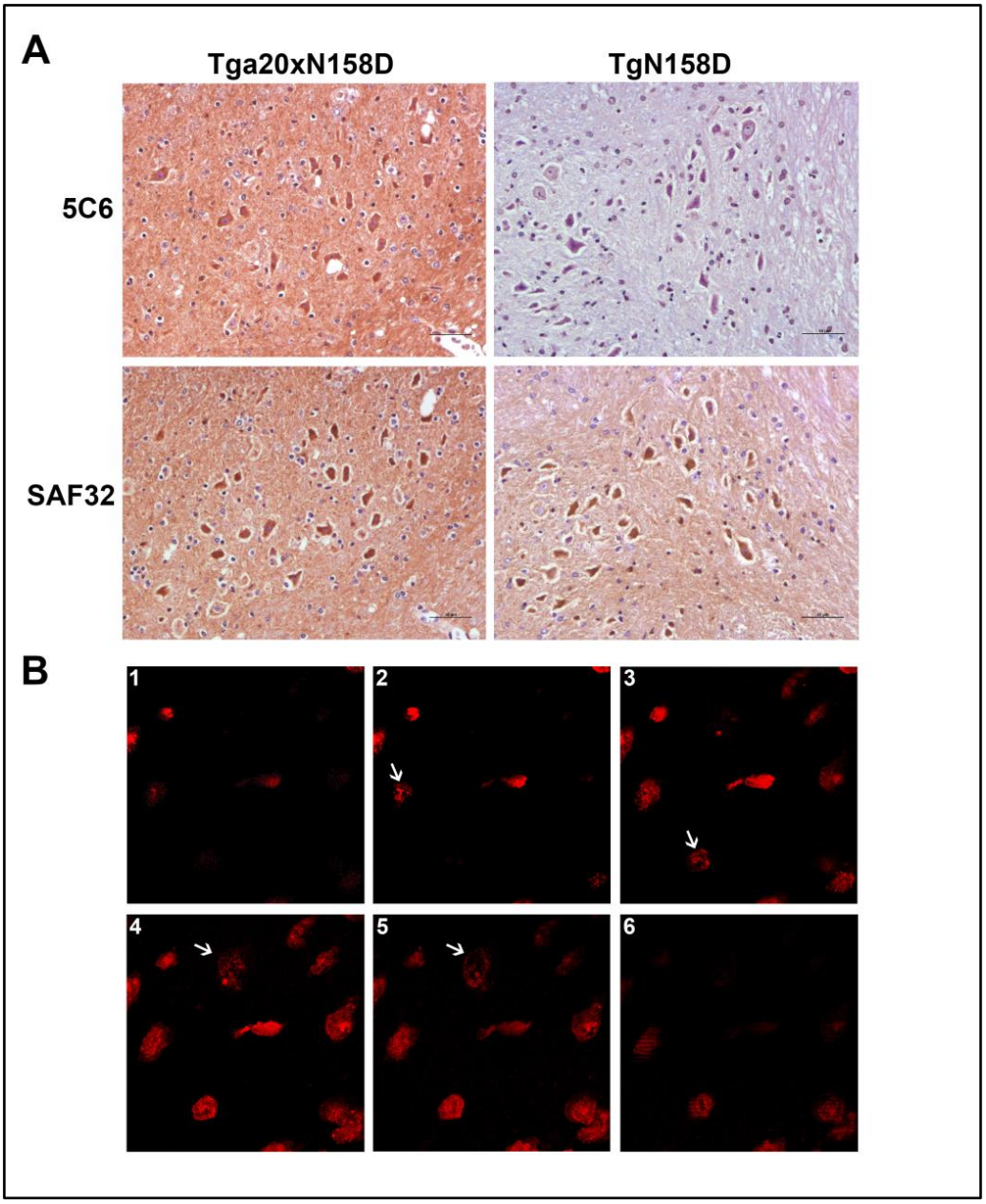
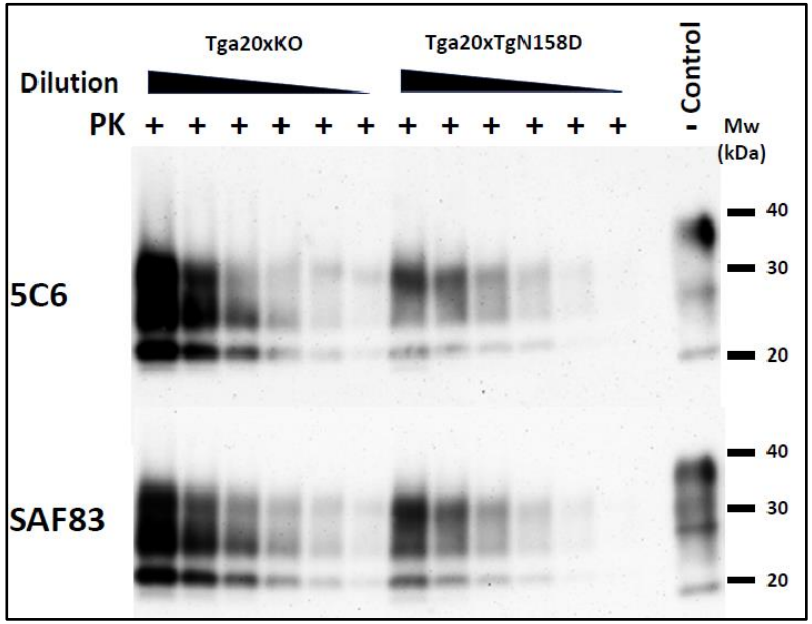
Supplementary material



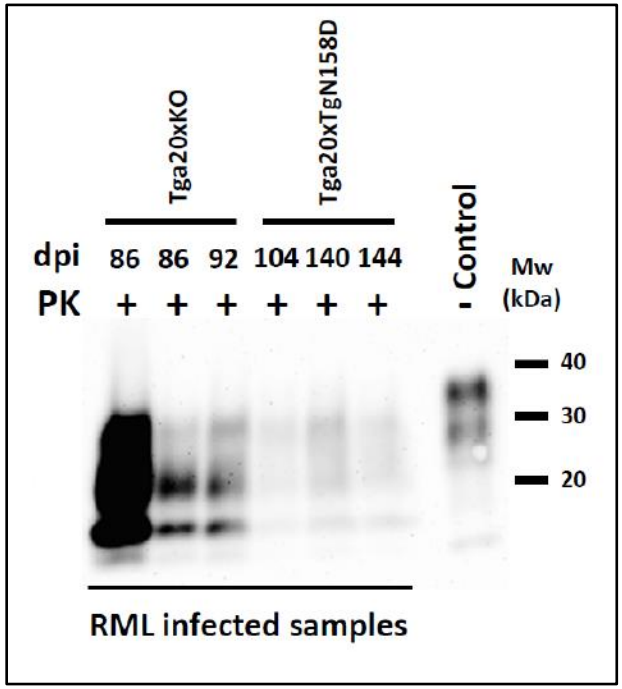
Supplementary figure 1: PrP expression levels from Tga20, Tga20xN158D, Tga20xKO and TgN158D mouse brains. 10% brain homogenates from Tga20, Tga20xN158D, Tga20xKO and TgN158D mice were diluted 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280 and were analyzed by Western blot using monoclonal antibodies 5C6 (1:2000) which does not bind N158D PrP, and SAF83 (1:400) able to bind both types of proteins. The N158D PrP from Tga20xN158D and TgN158DxTgN158D mice is not observed when 5C6 monoclonal antibody is used but the use of SAF83 shows how the PrP expression levels of Tga20, Tga20xN158D, Tga20xKO and TgN158D are approximately 8x, 4x+2x, 4x+0x and 4x compared to wild type mouse PrP^{C}, respectively based on signal intensity. No significant differences are observed in the electrophoretic migration patterns. Mw: Molecular weight.



Supplementary figure 2: Histological localization of PrP^C in Tga20xTgN158D and TgN158D mouse brains. (a) Immunohistochemical detection of PrP^C in neurons of the deep cerebellar nuclei from a Tga20xN158D and a TgN158D mouse using 5C6 and SAF32 monoclonal antibodies. 5C6 antibody produces intense immunostaining in the Tga20xN158D mouse brain, corresponding to a mouse wt PrP staining, since this antibody does not recognize N158D PrP. Accordingly, no immunostaining is observed in the TgN158D mouse using the same antibody. However, SAF32 produces a strong immunolabeling in a serial histological section from the same animal showing the distribution of N158D PrP. Similar immunolabeling is observed between Tga20xN158D and TgN158D mice using SAF32 antibody. **(b)** TgN158D mouse brain serial optical z-sections by confocal microscopy. To more clearly determine the localization of N158D PrP, fluorescence emission from a TgN158DxTgN158S mouse brain, stained using SAF32 antibody, was analyzed using confocal microscopy. Serial 0.5µm z-sections of medulla oblongata from this mouse were obtained using a green helium/neon (543 nm) laser system. A very intense neuronal staining of N158D PrP is observed. N158D PrP was detected in the neuronal membrane (arrows).



Supplementary figure 3: PrP^{res} detection from 22L inoculated Tga20xKO and Tga20xN158D mouse brains using a monoclonal antibody unable to bind N158D PrP. 10% brain homogenates from 22L inoculated Tga20xKO and Tga20xN158D mice were digested with 80 µg/ml of Protease-K (PK) and then diluted 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. Digested samples were analyzed by Western blot using two monoclonal antibodies subsequently: first, 5C6 (1:2000) which does not bind N158D PrP, and later, using the same membrane, SAF83 (1:400) able to bind both types of proteins. No significant differences are observed between both blots indicating that N158D PrP was not converted at least at the level to be distinguished by this technique. Control: Undigested Tga20xKO brain homogenate. Mw: Molecular weight.



Supplementary figure 4: PrP^{res} detection from RML inoculated Tga20xKO and Tga20xN158D mouse brains. 10% brain homogenates from RML inoculated Tga20xKO and Tga20xN158D mice, selected with different days post inoculation (dpi) were digested with 80 µg/ml of Protease-K (PK). Digested samples were analyzed by Western blot using SAF83 (1:400) and the signal level of PrP^{res} are compared. Despite the RML inoculated Tga20xKO mice were culled at ~90 dpi, the amounts of PrP^{res} are significant higher than the observed in the RML inoculated Tga20xN158D mice. This result suggests that the elongation of the incubation times in this model is likely due to a slower conversion of the wild-type PrP^{C}. Control: Undigested Tga20xKO brain homogenate. Mw: Molecular weight.

ESTUDIO 2

A single amino acid substitution, characteristic of mammals poorly susceptible to prion diseases, delays the propagation of different prion strains in highly susceptible transgenic models

A single amino acid substitution, characteristic of mammals poorly susceptible to prion diseases, delays the propagation of different prion strains in highly susceptible transgenic models

Abstract

Specific amino acid variants of PrP are key factors determining susceptibility to prion diseases. We previously evidenced the resistance conferred by a unique amino acid substitution of canids when expressed in the mouse PrP. We further corroborated the protective effect of this mutant PrP by demonstrating its dominant-negative effect against a variety of infectious prion strains of different origins and characteristics. Here, we show that the expression of this single amino acid change significantly increases the survival time in transgenic mice expressing bank vole PrP^C, which is inherently prone to misfolding, when inoculated with different prion strains (CWD-vole strain and an atypical strain of spontaneous origin). Our results indicate that this amino acid substitution is able to confer a protective effect against the propagation of notably different strains, even when expressed in a PrP^C uniquely susceptible to a wide range of prion isolates. Together with our previous reports, these results reinforce the importance of this residue as a candidate in the development of therapeutic approaches against prion diseases.

Introduction

Prions are self-propagating infectious proteins producing fatal neurodegenerative disorders known as transmissible spongiform encephalopathies (TSE) or prion diseases. These pathologies, characterized by spongiform changes, gliosis and neuronal degeneration in the central nervous system (CNS) of affected individuals, include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids and Creutzfeldt-Jakob disease (CJD) in humans (Collins *et al.*, 2004; Colby and Prusiner, 2011). Although prion diseases can have a sporadic, genetic or infectious origin (Prusiner, 1998a), the main event in the pathogenesis of these disorders is the posttranslational conversion of the normal cellular prion protein (PrP^C), into a protease resistant, β-sheet-rich isoform termed PrP^{Sc} which accumulates in the CNS leading to neurodegeneration (Prusiner, 1982).

It was previously found that the expression of aspartic acid (D) at codon 163, one particular PrP^C polymorphism exclusive of Canidae family (Stewart *et al.*, 2012), could

be the key to the unusual resistance of canids to prion diseases (Fernandez-Borges *et al.*, 2017b). Studies using recombinant proteins in the presence of denaturing agents and both *in vitro* and *in vivo* prion propagation studies aimed at assessing the susceptibility of species historically considered as prion-resistant (i.e. leporids, equids and canids) have demonstrated that dog PrP^C shows the highest resistance to misfolding (Polymenidou *et al.*, 2008; Khan *et al.*, 2010; Chianini *et al.*, 2012; Vidal *et al.*, 2013; Bian *et al.*, 2017). By contrast, the bank vole (*Myodes glareolus*) is probably the species at the opposite end of the mammalian susceptibility scale to prion diseases. Bank voles are rodents which have proven to be highly susceptible to prion infection, being widely used in prion research since they can efficiently propagate a broad spectrum of prion strains (Nonno *et al.*, 2006; Agrimi *et al.*, 2008; Di Bari *et al.*, 2013; Watts *et al.*, 2014; Pirisinu *et al.*, 2016). Bank vole PrP^C is polymorphic, expressing either methionine (M) or isoleucine (I) at codon 109 (Cartoni *et al.*, 2005). The adaptation of CWD to voles carrying I at codon 109 led to the isolation of the fastest known prion strain so far, characterized by survival times of ~35 days (Di Bari *et al.*, 2013). Interestingly, it was also found that the overexpression of bank vole I109 PrP in transgenic mice led to the development of a spontaneous TSE, making them a very useful model for studying sporadic prion diseases (Watts *et al.*, 2012). The development and the study of models of these forms of prion diseases are essential since the origin of sporadic TSE is still elusive. However, it has been suggested that sporadic prion diseases could be attributable to a random and stochastic misfolding of PrP^C into PrP^{Sc}, which subsequently accumulates, producing the clinical and neuropathological features associated with prion disorders (Will and Ironside, 2017).

As aforementioned, it was found that dog D163 PrP^C substitution is probably the main determinant of the resistance of canids to prion diseases. The presence of this single substitution in mouse PrP^C (N158D substitution in mouse PrP^C numbering) was able to impede the prion propagation both *in vitro* and *in vivo* (Fernandez-Borges *et al.*, 2017b). We further demonstrated the ability of N158D PrP^C to act as a dominant-negative protein *in vivo*. The coexpression of this mutant PrP^C variant and wild-type mouse PrP^C significantly delayed the onset of the disease in transgenic mice inoculated with several prion strains of different origins and characteristics, making N158D PrP^C a promising candidate in the search for effective dominant-negative proteins against a broad spectrum of prion strains (Otero *et al.*, 2017). In the present study, we aimed at determining whether

this substitution could also prevent or delay the onset of the prion disease in a highly susceptible model. Thus, we inoculated transgenic mice overexpressing bank vole I109 PrP and carrying this specific residue (TgVole-N159D mice), with two prion isolates, and the survival times obtained in these mice were compared with those exhibited by transgenic mice expressing comparable levels of bank vole PrP^C (TgVole mice) inoculated with the same isolates. To corroborate the ability of the amino acid substitution to exert a protective effect against prions of very different features the isolates used herein were a classical prion strain: CWD-vole (Di Bari *et al.*, 2013), and an atypical prion isolate: Sp-TgVole inoculum. Sp-TgVole is an atypical, infectious strain originated from non-inoculated TgVole mice, which develop a spontaneous TSE due to the overexpression of bank vole I109 PrP. Survival periods obtained in the bioassay were also correlated with those shown by non-inoculated, spontaneously sick TgVole-N159D and TgVole mice. We observed that TgVole-N159D mice presented from 52 to 108% longer survival periods than TgVole mice with both inoculated strains. These results agree with our previous research, demonstrating that the expression of this specific amino acid change in PrP is able to interfere with prion propagation even when expressed in a PrP^C highly susceptible to misfolding, delaying the onset of prion diseases of very different characteristics.

Materials and Methods

Ethics statement

All procedures involving animals were approved by the Ethics Committee for Animal Experiments of the University of Zaragoza (permit number PI32/13) and were performed in accordance with the recommendations for the care and use of experimental animals and in agreement with Spanish law (R.D. 1201/05).

Inoculation of transgenic mouse models and sample processing

Two different transgenic mouse models were used in the present study: transgenic mice expressing ~3-4x the I109 polymorphic variant of bank vole PrP and carrying the critical dog amino acid substitution (I109-N159D PrP^C), hereafter referred to as TgVole-N159D mice, and mice overexpressing ~3-4x bank vole I109 PrP^C, hereafter referred to as TgVole mice, which were used as controls. The murine *PRNP* promoter was used for both I109-N159D and I109 PrP^C expression, in a murine $Prnp^{0/0}$ background, and were

generated and characterized similarly to what has been previously described (Castilla *et al.*, 2004a).

Mice were intracerebrally inoculated under isoflurane anesthesia into the right cerebral hemisphere with 20 µl of a 10% brain homogenate. Two different isolates were inoculated in both TgVole-N159D and TgVole mice: CWD-vole, a CWD strain adapted to bank voles expressing I109 PrP, characterized by unprecedented short survival times (Di Bari *et al.*, 2013) and an atypical prion isolate of spontaneous origin (Sp-TgVole isolate). Sp-TgVole inoculum was obtained from brain homogenates of TgVole mice which had developed the spontaneous neurodegenerative disorder linked to the overexpression of the I109 variant of bank vole PrP at 187±25 days of age. Intracerebral injections were performed using a 50-µl precision syringe and a 25-G needle. After the inoculation, a dose of buprenorphine (0.3 mg/kg) was subcutaneously administered to achieve analgesia.

Following inoculation, animals were monitored for onset of neurologic signs. Mice were culled by cervical dislocation when clinical signs of terminal disease were detected (i.e. severe locomotor disorders, poor body condition and any impairment in their ability to feed) and their brains were collected. Coronal sections at the level of frontal cortex and medulla oblongata were separated and immediately stored at -80ºC for biochemical analyses. The remaining brain tissue was placed in 10% formalin fixative for subsequent histological studies.

Histopathological evaluation

Brains fixed in formalin were transversely cut at four standard levels for the neuropathological study of the following nine brain areas: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellum (Cbl) and medulla oblongata (Mo) (Fraser and Dickinson, 1968). Formalin-fixed brain tissues were embedded in paraffin wax, and 4-µm tissue sections were mounted on microscope slides and stained with hematoxylin and eosin. The intensity and distribution of spongiform changes were blindly evaluated using an optical microscope (Zeiss Axioskop 40) and semiquantitatively scored on a scale of 0 (absence of lesions) to 5 (high intensity lesions) in each of the aforementioned brain regions.

Analysis of PrP^{Sc} deposition

The detection of PrP^{Sc} deposits in paraffin-embedded brains was performed using the paraffin-embedded tissue (PET) blot technique, as formerly described (Schulz-Schaeffer *et al.,* 2000; Andreoletti *et al.,* 2004). PrP^{Sc} immunodetection was performed using Sha31 primary monoclonal antibody (1:8000; SPI-Bio) followed by an incubation with an alkaline phosphatase-coupled goat anti-mouse antibody (1:500; Dako). Immunolabeling was revealed using the NBT/BCIP substrate chromogen (nitro blue tetrazolium/5 –bromo-4-chloro-3-indolyl-phosphate; Sigma-Aldrich). The presence, intensity and distribution of PrP^{Sc} aggregates were evaluated using a Zeiss Stemi DV4 stereomicroscope and semiquantitatively scored as described for spongiform lesions.

Additionally, the distribution of PrP^{Sc} deposition was analyzed by immunohistochemistry using several brain samples from TgVole-N159D and TgVole mice inoculated with the Sp-TgVole strain. Brains from spontaneously sick TgVole-N159D and TgVole mice, from which the Sp-TgVole source was obtained, were used as controls. The protocol used was similar to that previously described (Monleon *et al.*, 2005). Paraffin-embedded sections were pre-incubated with 98% formic acid for 5 min followed by hydrated autoclaving at 96ºC in citrate buffer for 20 min. Peroxidase activity was blocked for 5 min using a peroxidase blocking reagent (Dako). Immunodetection of PrP^{Sc} was performed using 6H4 monoclonal antibody (1:100, Prionics) followed by an anti-mouse Envision polymer (Dako). Sections were subsequently incubated with DAB (diaminobenzidine, Dako) and counterstained with hematoxylin.

Data analysis

Survival times were analyzed with the Kaplan-Meier method and the obtained survival curves were compared between mice carrying the N159D substitution and controls using the log rank test (α= 0.05). Differences in histopathological and PrP^{Sc} deposition profiles between different transgenic mouse models were analyzed using the nonparametric Mann-Whitney *U* test and considered significant at $p<0.05$. Data analysis, Kaplan Meier curves and histopathology graphics were performed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

Results

Expression of the N159D PrP^C substitution strikingly increases survival time with both CWD-vole and Sp-TgVole strains

Transgenic mice expressing bank vole I109 PrP^C and carrying the resistance-associated amino acid substitution from dog PrP^C [(TgVole-N159D mice (∼3x-4x)] were intracerebrally inoculated with the CWD-vole, or Sp-TgVole strains, the latter being of spontaneous origin.

TgVole mice expressing comparable levels of bank vole I109 PrP^C (∼3x-4x) were challenged with the same isolates and selected as controls. TgVole-N159D mice inoculated with the CWD-vole strain showed a great increase in the survival period of 108% as compared with TgVole mice, which developed the disease showing strikingly short survival times of 61±4 dpi. TgVole-N159D mice inoculated with the Sp-TgVole isolate presented a more discrete, but considerable, increment in the survival times of 52%, as compared to TgVole mice (Table 1). The relative increase in survival shown by Sp-TgVole inoculated TgVole-N159D mice was very similar to that observed for non-inoculated TgVole-N159D mice, which develop the spontaneous TSE showing an increase of 56% as compared with non-inoculated TgVole animals (Table 1). Significant differences in survival were obtained between TgVole-N159D and TgVole mice for both strains inoculated and between non-inoculated, spontaneously sick TgVole-N159D and TgVole mice (Figure 1).

Despite the significant delay in the appearance of clinical signs, TgVoleN159D mice developed clinical signs of neurodegeneration identical to those shown by TgVole mice. In CWD-vole infected animals we observed dorsal kyphosis, circling, cachexia and tremor. Mice inoculated with the Sp-TgVole isolate showed mild kyphosis and a rapidly progressive ataxia.

Table 1: Survival periods of TgVole and TgVole-N159D mice

Inoculum	Model	Attack rate[a]	Survival time (mean±SEM)[b]	Relative increase in survival time (%)[c]
CWD-vole	TgVole	5 [d] /5 (100%)	61±4	-
	TgVole-N159D	6/6 (100%)	127±13	108%
Sp-TgVole	TgVole	7/7 (100%)	120±9	-
	TgVole-N159D	5 /5 (100%)	182±3	52%
Non-inoculated [e]	TgVole	NA	187±25	-
	TgVole-N159D	NA	292±10	56%

[a] Data based on PrP^{res} detection.

[b] Survival times were calculated as the number of days between inoculation and euthanasia in the case of mice inoculated with CWD-vole, or Sp-TgVole isolates, provided that the mouse developed clinical signs consistent with a TSE. Survival times are expressed as mean (± SEM) number of dpi. For non-inoculated mice survival times were calculated as the mean age at which the animals developed the sporadic TSE. Survival times are expressed as mean (± SEM) days of age.

SEM, standard error of the mean; dpi, days post-inoculation. NA Not applicable

[c] Extension of the survival times in TgVole-N159D mice was calculated as the difference between the average survival time of TgVole-N159D and that of TgVole expressed in relative percentages to the average survival times of TgVole mice.

[d] One animal from the CWD-vole inoculation group died due to a concomitant disease during the initial stages of the study and was excluded from the analyses. This animal exhibited no spongiform lesions or PrP^{Sc} deposits and was not included in calculations of the SEM or attack rate

[e] Data provided by Dr. Joaquín Castilla

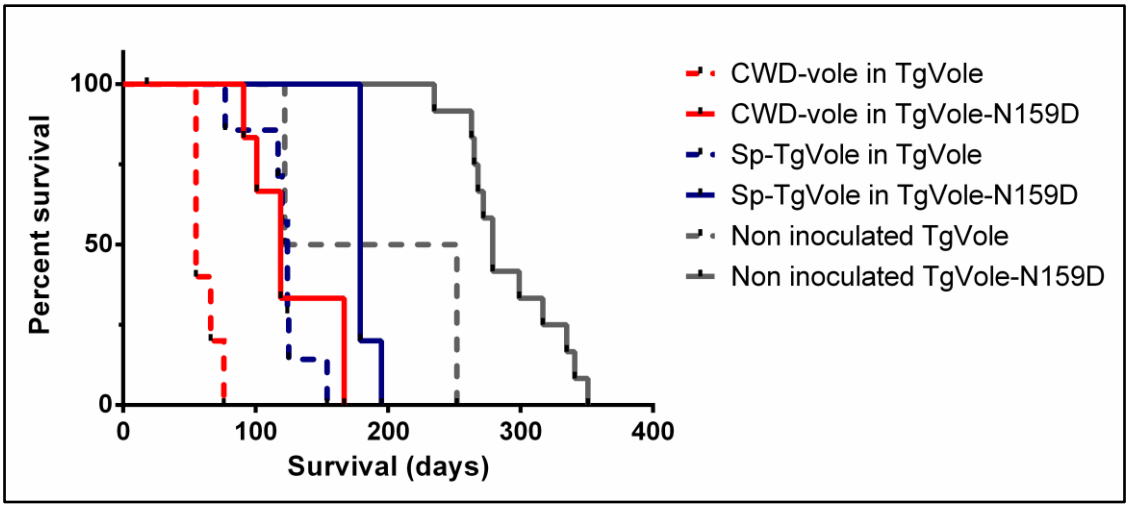


Figure 1: Survival curves for TgVole and TgVole-N159D mice challenged with CWD-vole, Sp-TgVole, or non-inoculated. Comparison of TgVole and TgVole-N159D survival curves using the log rank test (α=0.050) revealed significant differences for the CWD-vole (p=0.0007) and Sp-TgVole (p=0.0011) inoculation groups. Significant differences were also obtained between non-inoculated, spontaneously-affected TgVole and TgVole-N159D mice (p=0.0011). Survival periods are expressed as dpi for CWD-vole and Sp-TgVole inoculated animals and days of age at which the mice succumbed to the spontaneous TSE for non-inoculated animals.

Expression of N159D substitution did not alter the neuropathological features of the disease

As we observed in our previous study (Otero *et al.*, 2017), the expression of the resistance-associated key amino acid substitution from dog PrP^{C} (Fernandez-Borges *et al.*, 2017b), although producing a significant delay in the onset of clinical signs, did not cause any significant modification in the neuropathological features developed by TgVole-N159D mice. Spongiosis and prion protein deposition patterns were evaluated semi-quantitatively in the nine previously described areas and no significant differences were found between TgVole-N159D and TgVole mice inoculated with the same isolate (Figure 2). We found that all CWD-vole inoculated animals developed moderate spongiform changes and a discrete distribution of PrP^{Sc} deposits, which were especially conspicuous in the thalamic area, whereas other brain regions, such as the hippocampus, showed very low spongiosis and PrP^{Sc} deposition scores (Figures 2 and 3).

These neuropathological features coincide with those described for I109 PrP bank voles infected with the same strain, in which a prominent involvement of the thalamus has also been reported (Di Bari *et al.*, 2013). Moreover, either TgVole-N159D and TgVole mice inoculated with the atypical Sp-TgVole isolate developed severe vacuolar changes in cortices (Fc and Tc) and hippocampus, the brain area in which we observed the most intense PrP^{Sc} deposition with this isolate (Figures 2 and 3). To verify that Sp-TgVole strain had maintained its neuropathological characteristics after experimental transmission to TgVole and TgVole-N159D mice, we performed a histopathological evaluation of brain samples from several TgVole and TgVole-N159D animals that had developed the disease spontaneously at ~ 180 and ~235 days of age respectively. These animals exhibited a PrP^{Sc} deposition pattern almost identical to that shown by our mice inoculated with the Sp-TgVole isolate (Supplementary figure 1).

The presence of spongiform lesions and PrP^{Sc} deposits in TgVole and TgVole-N159D mice inoculated with Sp-TgVole strain confirms that these mice developed a prion disease which originated spontaneously in the same transgenic models and was transmitted.

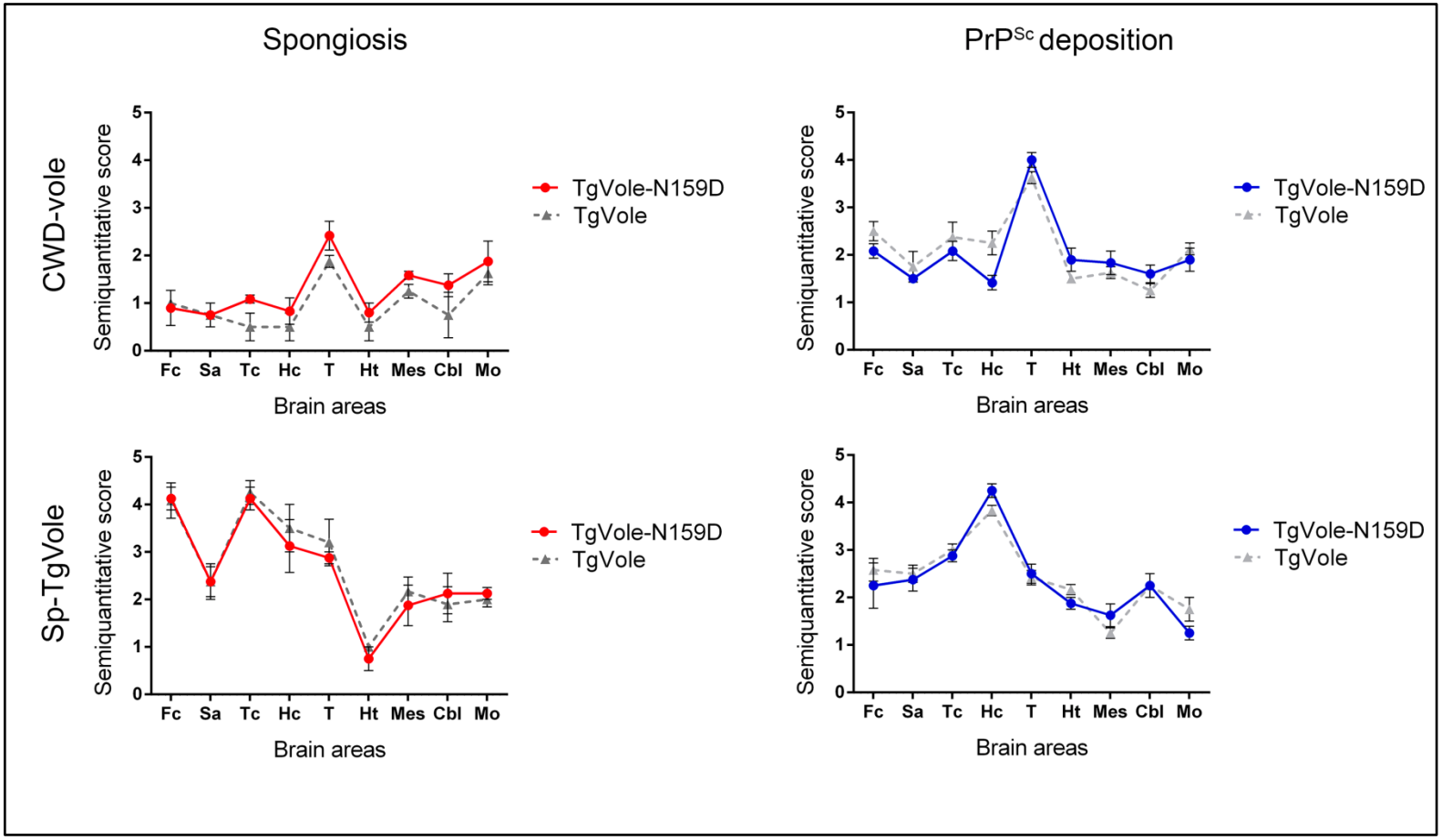


Figure 2: Spongiosis and PrP^{Sc} deposition profiles in the brains of TgVole and TgVole-N159D mice inoculated with different prion isolates. Spongiosis and PrP^{Sc} deposition were evaluated semiquantitatively on a scale of 0 (absence of lesions/deposits) to 5 (high intensity lesion/deposition) in the following nine brain areas: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellum (Cbl), and medulla oblongata (Mo). Comparison of the lesion and PrP^{Sc} deposition profiles of TgVole and TgVole-N159D mice revealed no significant differences between the lesion nor the PrP^{Sc} distribution and intensity (α=0.05, Mann-Whitney *U* test).

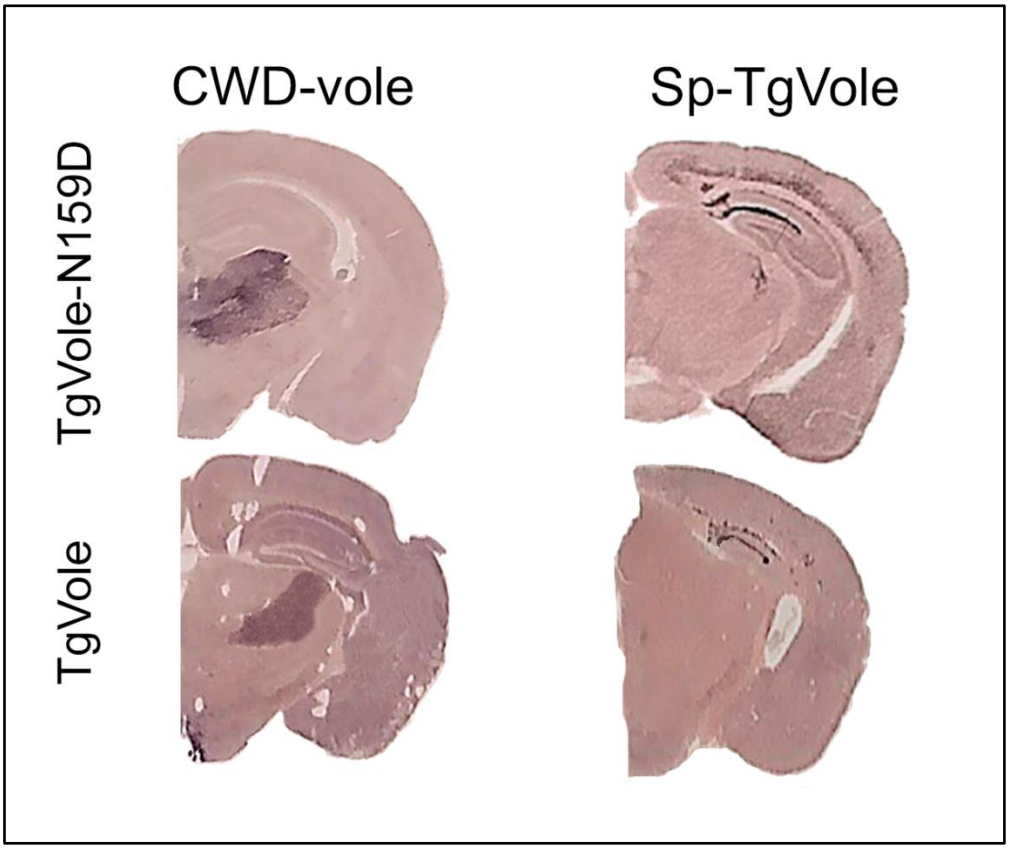


Figure 3: PET blot images of coronal brain sections from TgVole and TgVole-N159D mice inoculated with the CWD-vole, or Sp-TgVole strains. The PrP^{Sc} deposition (dark purple) distribution of mice expressing the N159D substitution is very similar to that of TgVole controls. Animals inoculated with the Sp-TgVole strain show a marked PrP^{Sc} deposition in the hippocampus. By contrast, in CWD-vole inoculated animals, the hippocampus shows a much weaker immunolabeling, with the thalamus being the most involved area. mAb: Sha31, 1:8000.

Discussion

Some specific naturally-occurring amino acid variants of PrP^{C} have been deeply studied regarding its relationship with resistance to prion diseases. Given the low susceptibility of scrapie infection when sheep possess at least one arginine at codon 171 (Westaway *et al.*, 1994b; Clouscard *et al.*, 1995), the protective effect of this specific residue in the propagation of prions has been evaluated in several studies, revealing also the dominant-negative inhibition of prion replication that this single amino acid substitution exerts both *in vitro* and *in vivo* (Kaneko *et al.*, 1997b; Zulianello *et al.*, 2000; Perrier *et al.*, 2002; Geoghegan *et al.*, 2009). The protective effect that heterozygosity at specific polymorphisms of human PrP^{C} provides against acquired, sporadic and some

familial prion diseases, is also widely known (Palmer *et al.*, 1991; Shibuya *et al.*, 1998). Among the resistance-associated human polymorphisms, E219K and G127V have shown not only to provide a strong protection against human prion diseases, but also to exert a dominant-negative inhibition on prion propagation when coexpressed with wild-type prion protein (Shibuya *et al.*, 1998; Perrier *et al.*, 2002; Asante *et al.*, 2015). Thus, the possible dominant-negative effect that the introduction of these naturally-occurring single mutations in an exogenous PrP^C could have, has been evaluated as an attractive hypothesis in the search for therapeutic approaches against prion diseases (Perrier *et al.*, 2002; Asante *et al.*, 2015). However, when looking for dominant-negative PrPs, it should be considered that not only is the *PRNP* genotype but also the infectious strain that determine susceptibility to TSE (Hill *et al.*, 2000; Collinge, 2001). The resistance to prion propagation provided by most of these polymorphisms, although strong, has proven to be strain-specific (Houston *et al.*, 2003; Atarashi *et al.*, 2006; Striebel *et al.*, 2011). Therefore, we decided to explore the potential protective effect of D163 residue of PrP^C, a key amino acid almost exclusive to canids (Stewart *et al.*, 2012; Fernandez-Borges *et al.*, 2017b), which are species in which no natural TSE have been detected and whose PrP^C shows high resistance to be misfolded *in vitro* (Vidal *et al.*, 2013) and has never been demonstrated to misfold *in vivo* (Polymenidou *et al.*, 2008; Fernandez-Borges *et al.*, 2017b).

We observed that mice overexpressing a mutated prion protein carrying the N158D amino acid substitution were completely resistant to prion infection when inoculated with a variety of mouse-adapted prion strains (Fernandez-Borges *et al.*, 2017b). Moreover, we found that the coexpression of wild-type mouse PrP^C and the mutant PrP^C variant carrying this specific dog amino acid substitution produced a dominant-negative effect in the *in vivo* propagation of mouse-adapted prion strains of scrapie and BSE origins (Otero *et al.*, 2017). However, could this amino acid change, characteristic of the most resistant mammalian species, prevent the misfolding of a PrP^C known as a universal acceptor of prions? (Watts *et al.*, 2014). Herein, we observed that mice overexpressing bank vole PrP^C I109, whose misfolding ability is such that its single overexpression leads to the development of a spontaneous prion disease (Watts *et al.*, 2012), are not completely resistant to prion infection, but show a significant delay in the onset of clinical signs when the N159D substitution is present in their PrP^C. Thus, we can suggest that the protective effect of this amino acidic change characteristic of canids is stronger when expressed in the mouse PrP^C. However, it should be considered that bank

vole PrP^C is greatly prone to conversion, and allows the propagation of prion strains which are refractory to be transmitted in wild-type and transgenic mice (Nonno *et al.*, 2006; Di Bari *et al.*, 2008; Pirisinu *et al.*, 2016). Bank voles expressing the I109 polymorphic variant of PrP^C, have demonstrated being more susceptible to certain familial prion disorders than transgenic mice overexpressing the homologous PrP with the corresponding mutation that causes the disease in humans (Asante *et al.*, 2013; Pirisinu *et al.*, 2016). However, we have observed here that the N159D substitution is able to increase the survival time in a model that overexpresses a PrP^C that is easily misfolded by almost every prion strain. The prolongation in survival times was especially notable in TgVole-N159D mice inoculated with the classical prion strain: CWD-vole. Even considering that it is the fastest known infectious strain (Di Bari *et al.*, 2013) inoculated in a highly susceptible model, survival time in TgVole-N159D mice inoculated with the CWD-vole strain was 108% longer than that of TgVole mice. Although survival times were also significantly increased in TgVole-N159D mice inoculated with the atypical isolate of spontaneous origin Sp-TgVole, the increase was more moderate than in the case of TgVole-N159D mice inoculated with the strain CWD-vole (Table 1). Therefore, we can suggest that the protective effect of N159D substitution is not homogeneous among the strains, although considerable against all of them. These results agree with our previous research in mice coexpressing the protein with the dog specific substitution, in which we observed that the mutated protein appeared to inhibit the propagation of prions in a strain-specific manner (Otero *et al.*, 2017).

The neuropathological features observed in our TgVole-N159D mice were very similar to those seen in TgVole animals, with no significant differences observed regarding lesional and PrP^{Sc} deposition profiles (Figure 2). We observed that all mice inoculated with the Sp-TgVole isolate, which originated from the spontaneous misfolding of I109 PrP^C, showed almost identical neuropathological profiles. Their profiles were also clearly distinguishable from those shown by mice inoculated with the CWD-vole strain (Figure 2 and 3). We further corroborated that Sp-TgVole inoculated mice showed the same distribution and morphology of PrP^{Sc} deposits than spontaneously sick TgVole-N159D and TgVole mice (Supplementary Figure 1). However, we should consider that both TgVole-N159D and TgVole mice develop a spontaneous TSE when they are not inoculated and, therefore, once they have exceeded the age at which this spontaneous disorder develops, we cannot know for certain if the neuropathology observed in the

animals is a consequence of this phenomenon, rather than an effect of the inoculation. We analyzed the ages at which Sp-TgVole inoculated mice developed disease and compared them with the data obtained from non-inoculated TgVole-N159D and TgVole mice. We observed that Sp-TgVole inoculated animals succumbed to disease at an earlier age than non-inoculated mice (Supplementary figure 2). Then, we can suggest that the inoculation of the Sp-TgVole isolate is causing a seeding acceleration phenomenon, which occurs when the transgenic model develops the disease spontaneously, but the pathological process can be accelerated by the exogenous inoculation of the agent (Fernandez-Borges *et al.*, 2013). Similar findings have been reported in a previous study using transgenic mice overexpressing bank vole I109 prion protein. It was found that the inoculation of brain extracts from spontaneously sick mice accelerates the onset of the disease but reproduces the same neuropathological hallmarks in transgenic mice expressing the same transgene (Watts *et al.*, 2012). In addition, we can suggest that the expression of the resistance-associated amino acid change, although significantly increasing the survival times, did not alter the pathological features of the inoculated strains. These results are in agreement with our previous findings (Otero *et al.*, 2017), and they suggest that the delay in the onset of clinical signs observed in TgVole-N159D mice is not caused by strain modifications produced by the N159D substitution.

The precise molecular mechanisms by which the N159D substitution produces an increase in survival times of TgVole-N159D mice remain unclear. However, we have observed that this residue is able to extend the survival period, not only in inoculated mice, but also in spontaneously sick animals. We can make this suggestion considering the differential survival times obtained in spontaneously sick TgVole (187±25 days) and TgVole-N159D mice (292±10 days) (Table 1 and Figure 1). Several theories have been proposed to explain the molecular basis of the protective effect that certain single amino acid changes in PrP^{C} exert on the propagation of prions. We previously demonstrated the complete protection provided by the N to D substitution in the mouse PrP context (Fernandez-Borges *et al.*, 2017b) and also the dominant negative effect of this protective mutation (Otero *et al.*, 2017). It has been suggested that, when certain PrPs carrying alleged protective mutations are in coexpression with wild-type PrP, the expression of these heterologous PrP molecules could interfere with the interaction between similar PrP monomers (Hope *et al.*, 1986; Prusiner *et al.*, 1990; Priola *et al.*, 1994). Therefore, this interference produced by heterologous molecules may produce a disruption of the

mechanism by which PrP^{C} is converted into PrP^{Sc} (Priola *et al.*, 1994) since allelic variants can be structurally incompatible (Jahandideh *et al.*, 2015). Actually, this mechanism has been successfully applied as a potential anti-prion therapy both *in vitro* (Horiuchi *et al.*, 2000) and *in vivo* (Seelig *et al.*, 2016). In addition, the introduction of single point mutations, and thus the heterologous interference caused by them, has also been proposed to provide an explanation for the long survival periods detected when a prion strain is transmitted to a new host (Priola *et al.*, 1994). N159D substitution may produce its protective effect causing alterations in the protein that reduce the rate of formation and the stability of newly formed fibrils (Fernandez-Borges *et al.*, 2017b). This pathogenic mechanism has been proposed before for similar amino acidic substitutions of PrP^{C} (Lee *et al.*, 2007; Asante *et al.*, 2015). In fact, it has been shown that the human PrP^{C} protective variant G127V, which also acts as a dominant-negative protein (Asante *et al.*, 2015), is unfavorable to form dimers and stable fibrils, thus having a protective effect in the development of prion diseases (Zhou *et al.*, 2016). Considering that it was observed that the substitution of N to D causes significant changes on the surface of PrP (Fernandez-Borges *et al.*, 2017b) we can suggest that the N159D substitution might also act in a similar way.

Together with our previous findings (Otero *et al.*, 2017), the present study indicates that the introduction of the specific N to D amino acid substitution of canids in an exogenous PrP^{C} not only provides complete resistance to TSE in certain models but is also able to significantly increase the survival times in models overexpressing PrPs highly susceptible to misfolding. In addition, N159D substitution delays the appearance of clinical signs of both infectious and spontaneous forms of prion diseases. Therefore, since we previously observed that the protein with the substitution acts as a dominant- negative against a great variety of strains of different origins, we can suggest that this protein could represent a promising approach in the search for genetic strategies against prion diseases.

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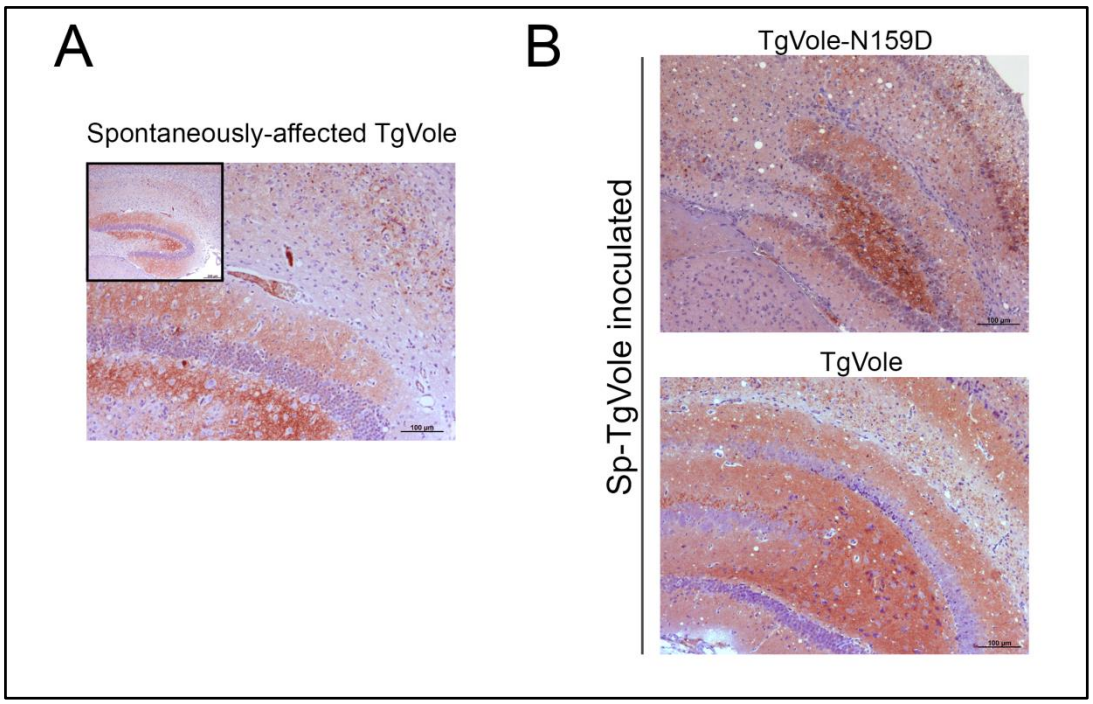
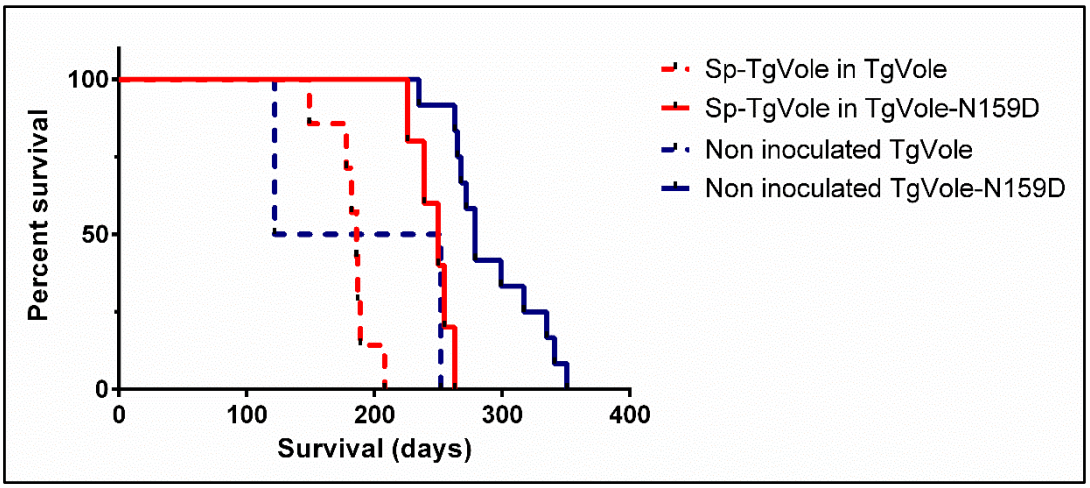
Supplementary material



Supplementary figure 1: Immunohistochemical analysis of a spontaneously affected TgVole mouse (A) and two Sp-TgVole inoculated mice of different PrP^{C} genotype (B). Note that the morphology and distribution of PrP^{Sc} deposits is almost identical in the three mice, all of them showing abundant granular PrP^{Sc} deposition in the dentate gyrus and the Ammon´s horn of the hippocampus. Immunodetection was performed using 6H4 monoclonal antibody (1:100).



Supplementary figure 2: Survival curves for Sp-TgVole inoculated mice and non-inoculated, spontaneously-affected mice. Survival periods are expressed as days of age at which the mice succumbed to disease. Sp-TgVole inoculated animals developed the disease at an earlier age than non-inoculated mice of the corresponding genotype.

ESTUDIO 3
N-attached glycans are not indispensable for the maintenance of human transmission barrier for prions or the preservation of BSE strain properties

N-attached glycans are not indispensable for the maintenance of human transmission barrier for prions or the preservation of BSE strain properties

Abstract

The implication of the glycosylation status of PrP^C in the conversion to its pathological counterpart and on cross-species transmission of prion strains has been widely discussed. Here, we have evaluated the effect of the glycosylation of human PrP^C on the transmission barrier for prion strains of different origins and how these strains behave when propagated over a human non-glycosylated PrP^C model. Classical and atypical scrapie, CWD, L-BSE, H-BSE, classical C-BSE, ovine and porcine-passaged BSE, and vCJD isolates were used as seeds/inocula in both *in vitro* and *in vivo* propagation assays using the TgNN6h mouse model (Haldiman T *et al*, 2013). On PMCA, only classical BSE prions propagated in the human non-glycosylated substrate, suggesting the maintenance of the human transmission barrier for prions. In addition, these isolates maintained the biochemical characteristics of BSE. On bioassay, except for vCJD, the direct inoculation of the original animal prion isolates did not cause disease in a first passage. However, all BSE PMCA-propagated prions were readily transmitted to TgNN6h mice, these results agreeing with our previous *in vitro* data. TgNN6h mice inoculated with PMCA-derived isolates or the vCJD direct isolate showed the characteristic neuropathological and biochemical hallmarks of BSE, suggesting that the absence of glycans did not alter the pathobiological features of BSE prions. Our results suggest that the glycosylation of human PrP^C is not essential for the preservation of the human transmission barrier for prions or for the maintenance of BSE strain properties.

Introduction

Prion diseases are fatal neurodegenerative disorders that include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids and Creutzfeldt-Jakob disease (CJD) in humans. Due to its association with the variant form of Creutzfeldt-Jakob disease (vCJD) in humans (Bruce *et al.*, 1997), BSE represented one of the major public health crises in Europe in the last decades. Several studies strongly suggest that the BSE epidemic was caused by a single strain, which can be transmitted to a wide range of hosts without apparent alteration of its pathobiological features (Brown *et al.*, 2003; Castilla *et al.*, 2003; Buschmann and Groschup, 2005; Green *et al.*, 2005). However, its pathogenicity to humans has proved to

be enhanced through passages in other species such as sheep and goats (Padilla *et al.*, 2011) or macaques (Lasmezas *et al.*, 2001).

A common feature of prion diseases is the accumulation of the pathological prion protein PrP^{Sc} in the central nervous system (CNS) of affected individuals. PrP^{Sc} is a self-propagating, misfolded isoform of the host-encoded cellular prion protein PrP^{C}, a membrane-anchored glycoprotein that is abundantly expressed in the CNS (Bolton *et al.*, 1982; Prusiner, 1982; Castilla *et al.*, 2004b). PrP^{C} sequence contains two consensus sites for N-glycosylation, involving asparagine residues at positions 181 and 197 in the human PrP sequence (or corresponding positions in other species) which can be variably occupied (Endo *et al.*, 1989), generating di-, mono-, and unglycosylated mature forms of PrP (Stimson *et al.*, 1999). Although this protein has a still-elusive and controversial function (Harris, 2003; Linden *et al.*, 2008) the expression of PrP^{C} in the cellular membrane is necessary for the development of the prion disease (Caughey and Raymond, 1991; Taraboulos *et al.*, 1992; Bueler *et al.*, 1993; Kaneko *et al.*, 1997a), and several studies have demonstrated the key role of N-linked glycans in the intracellular trafficking and membrane location of PrP^{C} (Rogers *et al.*, 1990; DeArmond *et al.*, 1997; Lehmann and Harris, 1997; Korth *et al.*, 2000; Salamat *et al.*, 2011). It was also shown that prion protein glycosylation can significantly modulate the interactions between heterologous PrP^{C} and PrP^{Sc} molecules, suggesting that glycans could be determining not only in the conversion efficiency of PrP^{C} into its pathological counterpart, but also in the cross-species transmission of prions (Priola and Lawson, 2001). In addition, in the context of a defined host, PrP^{C} can be misfolded into a great variety of prion strains, which are characterized by unique clinical features, neuropathological patterns and biological properties, including distinctive ratios of PrP^{Sc} glycoforms, which in general are faithfully recapitulated upon serial passaging in the same animal species (Somerville and Ritchie, 1990; Bruce, 1993; Collinge *et al.*, 1996; Bruce, 2003; Collinge and Clarke, 2007). Thus, the role of PrP^{C} glycosylation in the transmission barrier phenomenon and in the encoding of the strain-specific properties has been widely investigated *in vivo*.

In the present study, we assessed the behavior of a wide array of prion strains when propagated in a human non-glycosylated PrP^{C} model (TgNN6h mice) (Haldiman *et al.*, 2013), following both *in vitro* and *in vivo* approaches. Using protein misfolding cycling amplification (PMCA), only BSE prions (i.e. cattle C-BSE, sheep and pig-passaged BSE, and vCJD) propagated in the non-glycosylated human substrate, in a way

that was consistent with the existence of a transmission barrier phenomenon. All PMCA-propagated prions were readily transmitted to TgNN6h mice on bioassay, which developed the neuropathological and biochemical hallmarks of BSE. However, except for vCJD, the direct inoculation of the original BSE isolates did not cause disease in the non-glycosylated human PrP^{C} model in a first passage. Taken together, our results suggest that the absence of glycans alter neither the strength of the human transmission barrier for BSE nor the BSE strain pathobiological features.

Materials and methods

Original isolates

SSBP/1 inoculum (classical scrapie) was obtained from the brain of a terminally ill ARQ/ARQ sheep, and was supplied by the Veterinary Laboratories Agency (VLA), New Haw, Addlestone, Surrey, UK.

Atypical scrapie (Nor98) isolate was obtained from the brain of a diseased sheep, and provided by UMR INRA ENVT 1225, Interactions Hôtes Agents Pathogènes, École Nationale Vétérinaire de Toulouse, Toulouse, France.

CWD isolate was prepared from the brain of a CWD-affected mule deer, and supplied by the Department of Veterinary Sciences, University of Wyoming, Laramie, Wyoming 82070, USA.

C-BSE, L-BSE and H-BSE isolates were obtained from the brain of diseased cows, and provided by el Laboratorio Central de Veterinaria, Algete, Madrid, Spain.

Sheep BSE isolate (sBSE) was obtained from the brain of a BSE-affected ARQ/ARQ sheep and was supplied by UMR INRA ENVT 1225, Interactions Hôtes Agents Pathogènes, École Nationale Vétérinaire de Toulouse, Toulouse, France.

Pig BSE isolate (pBSE) was prepared from the brain of a terminally ill minipig, inoculated with sBSE (P-1224) (Hedman *et al.*, 2016), and was provided by Centro de Encefalopatías y Enfermedades Transmisibles Emergentes, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain.

vCJD isolate was supplied by Fundación Hospital Alcorcón de Madrid, Madrid, Spain.

All inocula were prepared from brain tissue as 1% (w/w) homogenates in PBS.

In vitro propagation of prions by PMCA

The *in vitro* prion replication and the PrP^{Sc} detection of amplified samples were carried out as described previously (Castilla *et al.*, 2005; Saa *et al.*, 2006). PMCA was performed using substrates based on brain homogenates from uninfected TgNN6h mice. The transgenic murine line TgNN6h (developed by Dr Qingzhong Kong, Case Western Reserve University, Cleveland, Ohio) expresses a mutated non-glycosylated human PrP^{C} carrying methionine at codon 129 (Haldiman *et al.*, 2013). After sacrifice by CO2 exposure, TgNN6h mice brains intended for substrate were perfused using PBS+5mM EDTA and immediately frozen at -80ºC. The brain substrate (10% brain homogenate) was prepared using a tissue grinder, homogenizing the brain tissue in PMCA buffer (PBS + NaCl 1% + 1% Triton X-100). PMCA was performed by mixing 5 µl of the corresponding inoculum with 50µl of substrate in 0.2 ml PCR tubes. Each inoculum was assayed in quadruplicate. Tubes were placed on the plate holder of a S-4000 Misonix sonicator (QSonica, Newtown, CT, USA) and subjected to incubation cycles of 30 min at 37ºC without shaking, followed by sonication pulses of 20s at 80% power. After a round of 24h PMCA, aliquots from the first round were diluted 1:10 in fresh TgNN6h substrate, being this procedure repeated for 15 rounds of PMCA. An equivalent number of unseeded tubes, containing only TgNN6h brain substrate, were exposed to the same procedure to control cross-contamination. BSE strains (i.e. cattle C-BSE, sheep and pig-passaged BSE, and vCJD) were further propagated for 10 more rounds in order to generate prions adapted to the TgNN6h substrate.

TgNN6h and Tg340 mice bioassay and sample processing

The original inocula and the *in vitro*-generated prion isolates during round 25 of PMCA (i.e. BSEC-PMCA, sBSE-PMCA, pBSE-PMCA and vCJD-PMCA inocula) were inoculated in both TgNN6h and Tg340 mouse models. The transgenic murine line TgNN6h expresses a mutated non-glycosylated human PrP^{C} (one-fold) in a murine *PRNP*-null background. This mutated human PrP^{C} contains two point mutations that

change asparagine to glutamine at residues 181 and 197, leading to the elimination of the two N-linked glycosylation sites. N181Q/N197Q PrP^{C} was generated from the human PrP-129M transgene by PCR mutagenesis and, although is unable to glycosylate during the post-translational processing, retains a normal intracellular trafficking and membrane localization, making TgNN6h mice susceptible to prion infection (Haldiman *et al.*, 2013). As controls, Tg340 mice, which express the normally glycosylated human 129M PrP^{C} (4-fold higher than in human brain) in a murine $PrP^{0/0}$ background (Padilla *et al.*, 2011), were inoculated with the same isolates.

Each mouse, under isoflurane anesthesia, received an intracerebral inoculation (20µl) into the right cerebral hemisphere, using a 50-µl precision syringe and a 25-G needle. To reduce postinoculation pain, each mouse was given a subcutaneous dose of buprenorphine (0.3 mg/kg). Following inoculation, animals were housed in cages placed in HEPA-filtered ventilated racks and monitored three times per week for onset of neurological signs of prion disease. Mice were culled by cervical dislocation when clinical signs of advanced TSE were detected (i.e. sustained kyphosis, severe ataxia, and poor body condition) or at the end of the study (600-700dpi). Brains were removed immediately after euthanasia and divided sagittally. One brain hemisphere was frozen at -80ºC for subsequent biochemical analyses. The other brain hemisphere was placed in 10% formalin fixative for up to 48h and used for neuropathological studies.

Neuropathology

Brains fixed in formalin were embedded in paraffin wax, cut into 4µm-thick sections and mounted on glass slides. For the evaluation of spongiform lesions, sections were stained with hematoxylin and eosin.

The morphology and brain distribution of PrP^{Sc} deposits were evaluated by immunohistochemical analysis using a similar protocol to that previously described (Monleon *et al.*, 2004). Briefly, after deparaffination and rehydration, sections were pre-treated with 98% formic acid for 10 min followed by incubation with proteinase K (4µg/ml¸F. Hoffmann La Roche) for 15 min. After hydrated autoclaving at 96ºC in citrate buffer for 20 min, immunodetection of PrP^{Sc} was performed by incubating the samples with 3F4 monoclonal antibody (1:1000, EMD Millipore, MAB 1562) for 30 min at room temperature followed by 30 min of incubation with an anti-mouse Envision polymer (Dako). DAB (diaminobenzidine, Dako) was used as the chromogen substrate.

Amyloid accumulation was also detected in several sections using the Congo Red-stain, as previously described (Zhang *et al.*, 2004). Briefly, deparaffinized and hydrated brain sections were incubated in an alkaline alcohol-saturated NaCl solution for 20 min following by incubation in a solution of 0.5% Congo Red prepared in an alkaline alcohol-saturated NaCl solution for 20 min. Sections were rinsed through two rapid changes of 100% ethanol and two changes of xylene, and then mounted with DPX.

Semiquantification of neuropathology results and data analysis

The intensity and distribution of spongiform changes and PrP^{Sc} immunolabeling were blindly evaluated using an optical microscope (Zeiss Axioskop 40) and semiquantitatively scored on a scale of 0 (absence of spongiosis or immunolabeling) to 5 (high intensity of lesion or immunolabeling) in the following brain regions: frontal cortex (Fc), thalamic cortex (Tc), occipital cortex (Oc), corpus callosum (Cc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellar cortex (Cbl), and medulla oblongata (Mo).

Kaplan Meier curves and histopathology graphics were performed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

Biochemical analysis of *in vitro*- and *in vivo*-generated prions

PMCA amplified samples and 10% brain homogenates from prion inoculated mice were digested using 170 μg/ml Protease-K (PK) during 1 h at 42°C with constant agitation (450 rpm) as previously described (Castilla *et al.*, 2005). Digestion was stopped by adding loading buffer (Laemmli buffer *NuPAGE*; Invitrogen Life Technologies) and the samples were analyzed by Western blot technique. Immunodetection of prion protein was performed with mouse monoclonal antibody 3F4 (1:10,000) and visualized using a horseradish peroxidase-conjugated secondary antibody and chemiluminiscence (Super Signal West Pico kit; Thermo Scientific Pierce).

Ethics statement

All animal experiments were approved by the Ethics Committee for Animal Experiments of the University of Zaragoza (permit number PI20/15) and were carried out in accordance with the recommendations for the care and use of experimental animals and in agreement with Spanish law (R.D. 1201/05).

Results

Non-glycosylated human PrP substrate is converted *in vitro* by C-BSE and BSE-derived isolates

In order to assess the *in vitro* misfolding ability of non-glycosylated human PrP and how the lack of glycans could affect the human transmission barrier for prions, TgNN6h mice brain homogenates were seeded *in vitro* with 9 prion strains of different origins. As aforementioned, classical BSE prions present particular abilities to cross species barriers (Sigurdson and Miller, 2003; Eloit *et al.*, 2005; Torres *et al.*, 2014), including that of humans (Bruce *et al.*, 1997), and, moreover, they show singular stable pathobiological features upon transmission to different hosts (Castilla *et al.*, 2003; Buschmann and Groschup, 2005; Green *et al.*, 2005). Taking these evidences into account, we divided the strains used in the present study in two groups, in a similar way to that previously described (Torres *et al.*, 2014): BSE-related prions and non-BSE related prions. BSE-related prions included cattle classical BSE (C-BSE), sheep and pig-passaged BSE and human vCJD, whereas sheep scrapie (SSBP/1 and atypical scrapie/Nor98), CWD, and atypical cattle BSE strains (L-BSE and H-BSE) were considered as non-BSE related prions. L-BSE and H-BSE strains were included in the group of non-BSE related prions since both strains present a pathology and epidemiology clearly distinct from C-BSE (Tranulis *et al.*, 2011).

Duplicates of four tubes were subjected to 15 serial PMCA rounds in an attempt to compare the ability of the abovementioned strains to generate misfolded non-glycosylated human PrP, which correlates with the number of serial PMCA rounds needed until protease resistant PrP (PrP^{res}) is detected. PrP^{res} positivity was evaluated through Western blot analysis. We detected that vCJD readily propagated in TgNN6h substrate, with a 100% of tubes being PrP^{res} positive after a single round of PMCA. Among the animal prion strains, only cattle C-BSE, sheep BSE (sBSE), and pig BSE (pBSE) propagated in TgNN6h substrate, showing different efficiencies, consistently with the existence of a transmission barrier. C-BSE propagated poorly in the non-glycosylated human substrate, with 25% of positive samples in the 15^{th} round, whereas sBSE skipped the barrier in the 7^{th} round showing 25% of positive tubes, and 75% PrP^{res} positive tubes from 11^{th} round. pBSE propagated in TgNN6h substrate in only 6 PMCA rounds (75% of tubes) and propagated in all replicates in the 9^{th} round (Figure 1A). When

analyzed by Western blotting, all isolates that were amplified showed a PrP^{res} single band at 19 KDa (Figure 1B).

We further propagated the isolates that were able to misfold TgNN6h PrP^{C} for 10 more rounds of PMCA, and these PMCA products were selected as challenge inocula *in vivo*: BSEC-PMCA, sBSE-PMCA, pBSE-PMCA and vCJD-PMCA inocula.

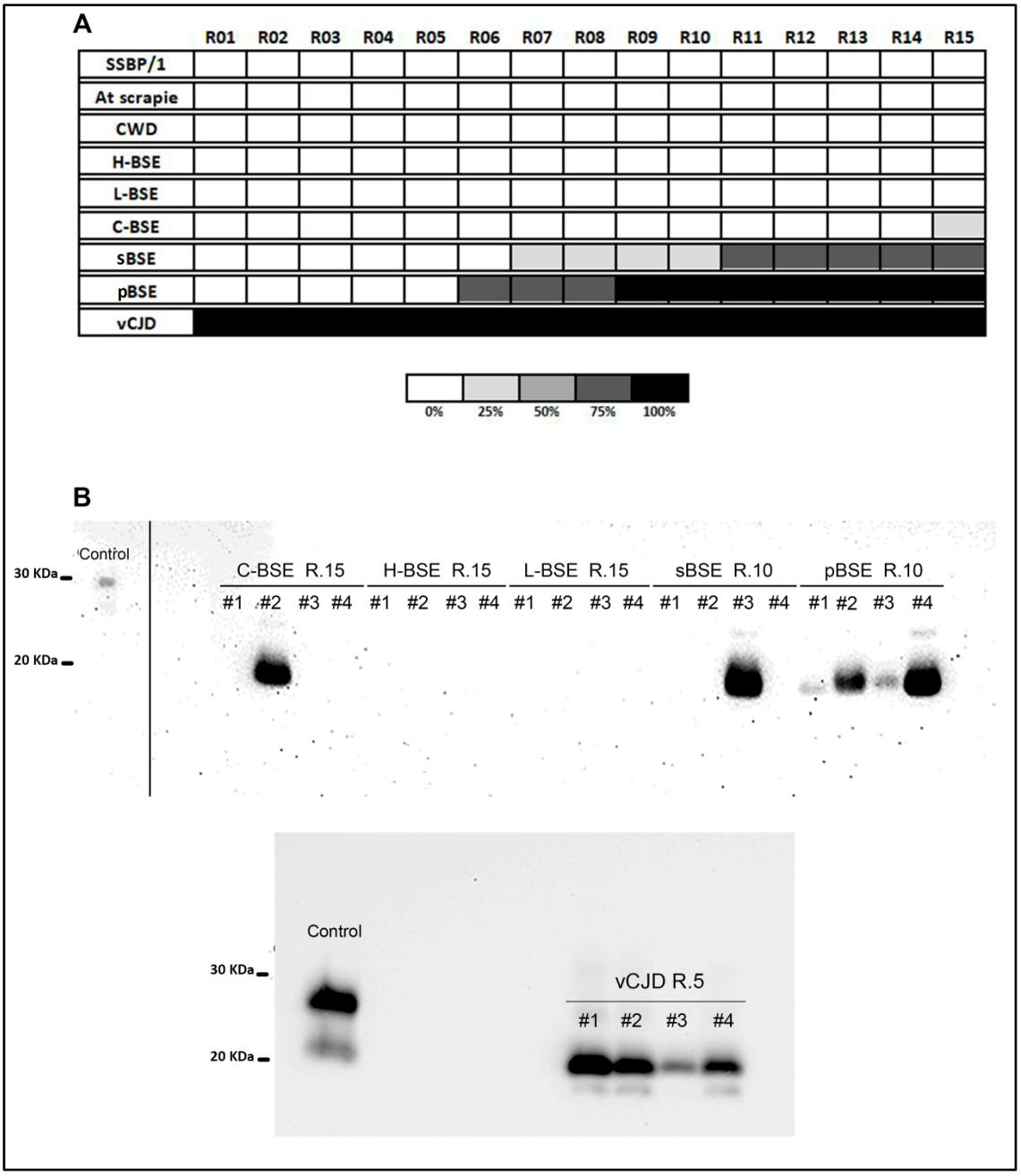


Figure 1: In vitro propagation assay. (A) Rounds (R01-R15) of serial PMCA using TgNN6h brain homogenate as substrate. The grey scale in the boxes below indicates the % of positive tubes (showing PK resistant TgNN6h PrP) out of the total number of tubes sonicated (n=4). **(B)** Biochemical analysis of TgNN6h PrP^{res} generated *in vitro* at round 15 (for C-BSE), round 10 (for sBSE and pBSE isolates), and round 5 (for vCJD). Seeded samples were digested with 170 μg/ml of proteinase K (PK) and analyzed by Western blot using monoclonal antibody 3F4 (1:10000). An unglycosylated band at 19 KDa is observed in C-BSE and sBSE seeded samples (one tube each) and in the four replicates seeded with pBSE. No amplification was detected in the tubes seeded with L-BSE or H-BSE isolates. Control: Undigested TgNN6h whole brain homogenate.

PMCA propagation of BSE isolates greatly facilitated the transmission to TgNN6h mice while direct inoculation requires longer incubation times.

Both original inocula and the PMCA-passaged, *in vitro*-adapted inocula were inoculated in two different animal models: TgNN6h and Tg340 mice. The TgNN6h transgenic line expresses approximately one-fold non-glycosylated human 129M PrP^{C} (Haldiman *et al.*, 2013), whereas the Tg340 transgenic line overexpresses human 129M PrP^{C} (at levels 4-fold higher than those detected in human brain) with the three possible glycoforms (di-, mono-, and unglycosylated) (Padilla *et al.*, 2011). At least 6 animals of each transgenic line were inoculated with each of the strains (Table 1).

To date, all TgNN6h mice inoculated with the *in vitro* propagated BSEC-PMCA, sBSE-PMCA, or pBSE-PMCA strains have succumbed to prion disease at first passage. Attack rates were of 100% in all cases, the transmission efficiency being evaluated by the appearance of clinical signs of TSE and/or by the presence of PrP^{res} in the brain (Figure 2). TgNN6h animals that developed clinical disease showed hyperesthesia, kyphosis and ataxic gait in a first stage of the disease, followed by weight loss, lethargy and ruffled coat in a later stage. These *in vitro* generated BSE strains transmitted to TgNN6h mice leading to similar survival periods, however, the shortest survival times were obtained in TgNN6h animals inoculated with the pBSE-PMCA strain (206±29 dpi), whereas sBSE-PMCA and BSEC-PMCA inoculated mice presented longer survival periods (222±24 and 279±24 dpi, respectively). Notably, no TgNN6h mouse inoculated with the direct (not PMCA-propagated) C-BSE, sBSE or pBSE isolates developed disease or accumulated detectable brain levels of PrP^{res} up to ~600 dpi (Table 1). However, after 584 dpi, one animal from the TgNN6h group challenged with the vCJD direct inoculum developed neurological signs, particularly tremor and ataxic gait. This animal and 5 further vCJD-inoculated TgNN6h mice that were culled at ~700 dpi with no signs of clinical disease, presented PrP^{res} accumulation in the brain, detected by both Western blot and immunohistochemical techniques (Table 1, Figure 2). At the moment of writing this document, several TgNN6h animals of the second passage of BSEC-PMCA (1 mouse, 227 dpi), sBSE-PMCA (4 mice, 218±5 dpi), and pBSE-PMCA (3 mice, 202±27 dpi) strains have developed clinical signs compatible with prion disease. PrP^{res} accumulation in the CNS of these mice will be further analyzed to confirm the transmission of the prion disease.

On the other hand, and in agreement with our previous *in vitro* results, the inoculation of the rest of animal prion strains (SSBP/1, atypical scrapie, CWD, L-BSE, and H-BSE) did not cause TSE disease nor accumulation of PrP^{res} in the brains of TgNN6h mice at first passage. In addition, to date, none of the Tg340 inoculated controls has developed disease with any of the strains inoculated at first passage.

These experiments are still ongoing; however, several inoculated groups of Tg340 mice have been culled at ~700 dpi, and no PrP^{res} accumulation has been detected in their brains (Table 1).

Table 1: Inoculation of TgNN6h and Tg340 mice with BSE-related and non-BSE related prions

Isolates	TgNN6h mice (1st passage)		Tg340 mice (1st passage)	
	Survival time (dpi)(mean ± SEM)[a]	Attack rate[b]	Survival time (dpi)[a]	Attack rate[b]
SSBP/1	698-750	0/12	592-727	0/6
Atypical scrapie	112-641	0/11	656-748	0/5
CWD	515-732	0/10	690-724	0/6
BSE-H	291-631	0/10	672[e]	*ongoing*
BSE-L	280-733	0/12	476[e]	*ongoing*
BSE-C	127-733[c]	0/11	488-711	0/6
sBSE	138-641[c]	0/11	419[e]	*ongoing*
pBSE	139-648[c]	0/9	721[e]	*ongoing*
vCJD	697±23	6/11 (55%)	476[e]	*ongoing*
BSEC-PMCA	279±24	11/11 (100%)	-	-
sBSE-PMCA	222±24	7[d]/7 (100%)	-	-
pBSE-PMCA	206±29	11/11 (100%)	-	-
vCJD-PMCA	216[e]	*ongoing*	-	-

[a] Survival times are shown as mean number of days between inoculation and euthanasia ± SEM, except when none of the inoculated mice developed clinical signs consistent with a TSE nor were found to be PrP^{res} positive. In the latter case, the survival periods of the first and last dead animal in each group are presented.

SEM standard error of the mean, *dpi* days postinoculation.

[b] Data based on PrP^{res} detection in the brain.

[c] Several animals from the BSE-C, sBSE, and pBSE inoculation groups were found dead through the bioassay without showing clinical signs. These animals were found to be PrP^{res} negative. The survival periods of the first and last dead animal in each group are presented.

[d] Three animals died due to intercurrent diseases during the initial stages of the bioassay and were excluded from the study. These animals were found to be PrP^{res} negative and were not included in calculations of the SEM or attack rate.

[e] Survival period of the mice at the time of writing this study.

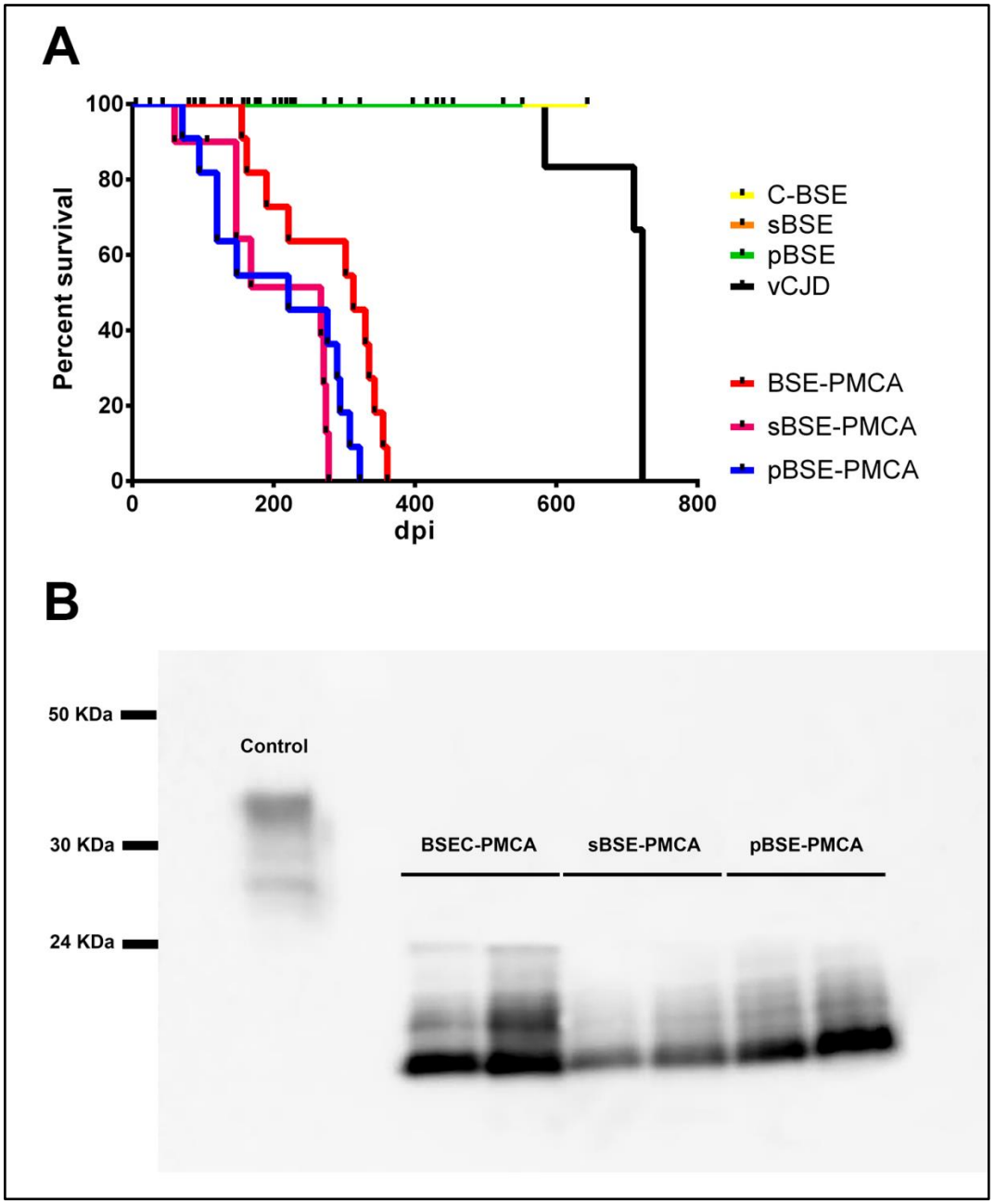


Figure 2: Bioassay in TgNN6h mice. (A) Survival curves for TgNN6h mice challenged with BSE-related prions at first passage. All animals inoculated with the *in vitro* propagated strains succumbed to prion disease whereas direct inoculation of C-BSE, sBSE, or pBSE strains did not cause disease at first passage. Only vCJD isolate transmitted to TgNN6h mice after the direct inoculation of the original isolate. **(B)** PrP^{res} detection from BSEC-PMCA, sBSE-PMCA, and pBSE-PMCA inoculated TgNN6h mice at first passage. 10% brain homogenates from challenged mice were digested with 170 µg/ml of PK and analyzed by Western blot using 3F4 antibody (1:10000). The glycoprofile shown by these mice is almost identical to that observed for the corresponding PMCA-propagated seeds, used as inocula for the bioassay. Control: Undigested TgNN6h 10% brain homogenate.

BSEC-PMCA, sBSE-PMCA, and pBSE-PMCA challenged TgNN6h mice developed very similar neuropathological features. The brain of these mice showed very intense spongiosis and abundant amyloid-like plaques surrounded by areas of severe spongiform change (florid-like morphology). Spongiform lesions were particularly intense in brain cortex (frontal, thalamic, and occipital cortex) and diencephalon, especially in the thalamus. Other brain areas, such as brainstem and cerebellum, presented much scarcer spongiform changes (Figure 3).

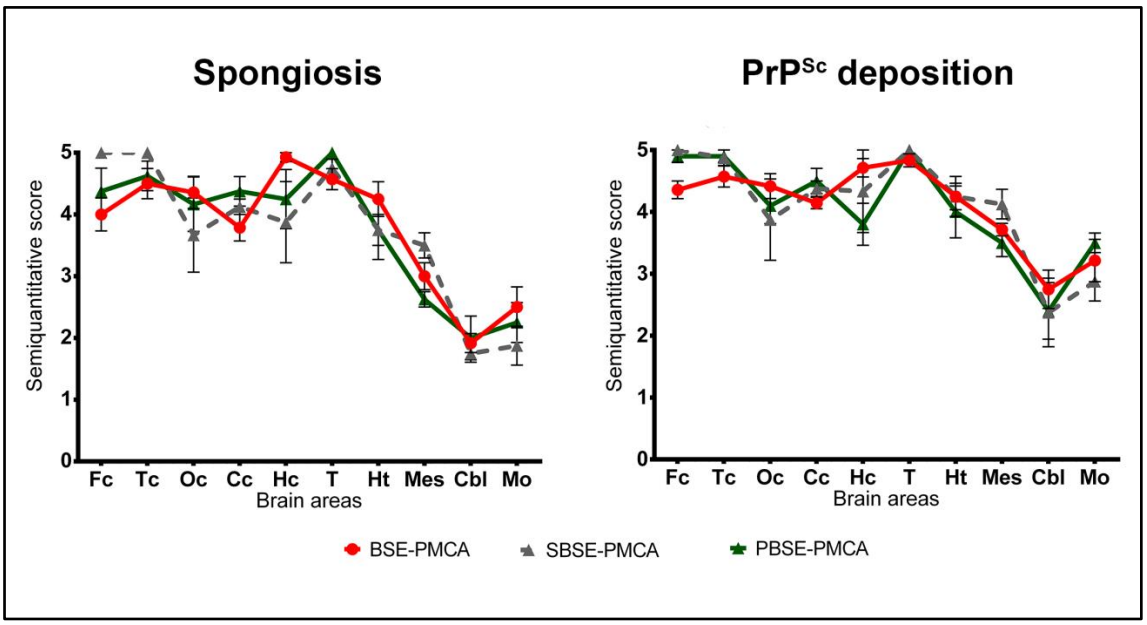


Figure 3: Spongiosis and PrP^{Sc} deposition profiles of TgNN6h mice inoculated with PMCA-propagated BSE isolates. Spongiform lesions and PrP^{Sc} deposition were evaluated semiquantitatively on a scale of 0 (absence of lesions/deposits) to 5 (high intensity lesion/deposition) in the following 10 brain areas: frontal cortex (Fc), thalamic cortex (Tc), occipital cortex (Oc), corpus callosum (Cc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellar cortex (Cbl), and medulla oblongata (Mo).

Immunohistochemical analysis revealed that the plaques which were observed with haematoxilin and eosin staining were composed of prion protein, presenting an amyloid core generally surrounded by a halo of spongiform degeneration (Figure 4 and Supplementary figure 1). These neuropathological changes are termed florid plaques and are characteristic of vCJD in humans (Will *et al.*, 1996; Ironside and Head, 2004). Large florid plaques were detected in the cerebral cortex, thalamus, hypothalamus, and white matter structures such as the corpus callosum. In certain brain areas, especially in the hippocampus, plaques were usually confluent and very disruptive; this precluded the

establishment of a reliable lesion profile in this brain region since a great variability was observed regarding vacuolization and PrP^{Sc} deposition between animals (Supplementary figure 2). In other areas, such as cerebellum and medulla oblongata, amyloid plaques were more discretely distributed. In addition to plaques, granular deposits of prion protein were detected throughout the brain.

vCJD-affected mice developed both florid plaques and large amyloid deposits

As aforementioned, only one out of 11 TgNN6h mice inoculated with the vCJD isolate developed neurologic signs compatible with a TSE, but Western blot and immunohistochemical analysis demonstrated PrP^{res} accumulation in the brains of 5 more vCJD-inoculated mice that were culled without clinical signs at more than 700 dpi. PrP^{res} positive, vCJD-infected mice developed sparse spongiform changes and PrP deposits, both substantially less intense than those observed in animals inoculated with the *in vitro* propagated BSE strains. Two of these mice, both culled without clinical signs at more than 700 dpi, showed only granular deposits of PrP^{Sc}, which were observed in the corpus callosum and the fimbria of the hippocampus. The other 4 PrP^{res} positive mice, including the mouse that was culled showing clinical signs, accumulated both granular PrP^{Sc} deposits and plaques. These mice presented florid plaques, but they were scant and confined to the subcallosal area (Figure 5A). Surprisingly, these 4 mice also showed large and dense PrP-positive deposits in the vicinity of the corpus callosum. Although these PrP-positive deposits were plaque-like, their morphology and size were significantly different from those of the florid plaques also found in these mice since they presented a multicentric morphology and a much larger size. Because these deposits appeared to be PrP^{Sc} plaques, brains of vCJD-positive mice were stained with Congo Red to test for amyloid plaque formation. The core of these dense deposits, pale in H-E, stained positively with Congo Red, confirming that they were composed of amyloid (Figure 5B).

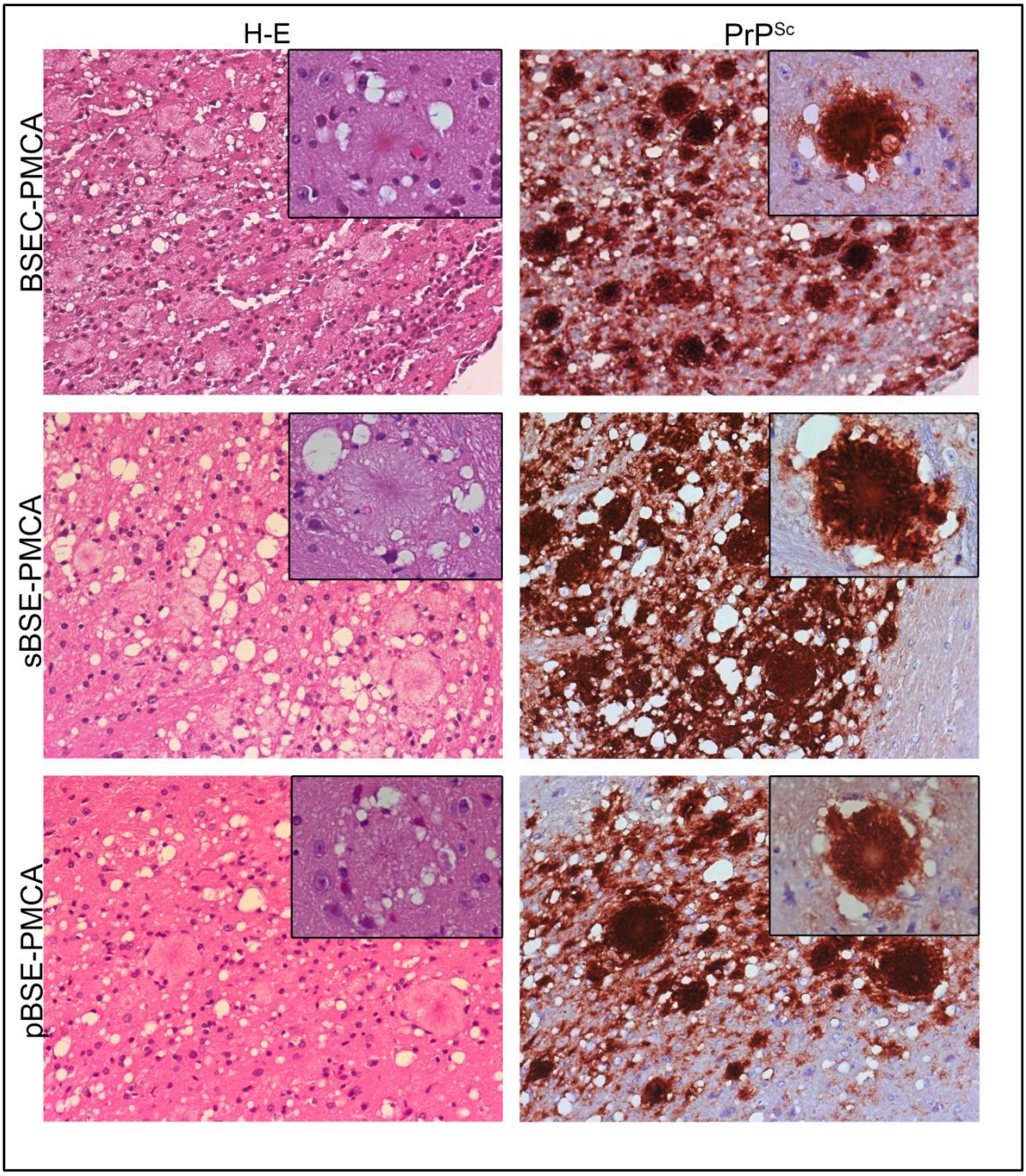


Figure 4: Spongiform changes and PrP^{Sc} deposits observed in TgNN6h mice inoculated with PMCA-propagated BSE isolates. Haematoxylin-eosin staining and immunohistochemical analysis of the brains of BSE-PMCA, sBSE-PMCA, or pBSE-PMCA affected mice showed the presence of conspicuous plaque-like deposits, surrounded by areas of severe spongiform change (florid plaques). (x20, insert pictures: x40). Immunohistochemistry was performed using the 3F4 antibody (1:1000). Brain areas: Thalamic cortex (BSEC-PMCA), mesencephalon (sBSE-PMCA), and thalamus (pBSE-PMCA).

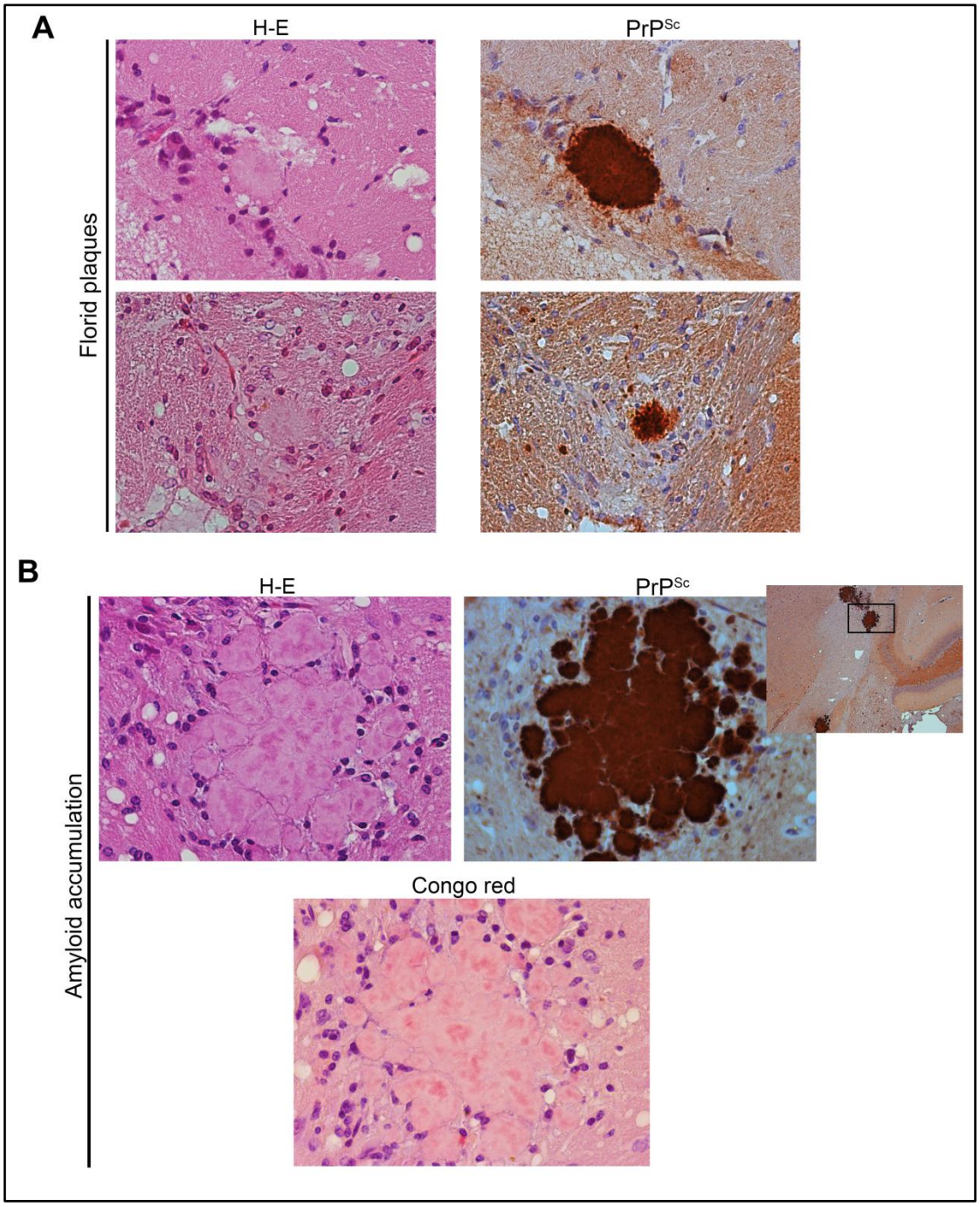


Figure 5: Histological changes and PrP^{Sc} deposits observed in TgNN6h mice inoculated with vCJD direct isolate. (A) Haematoxilin and eosin staining and immunohistochemical analysis of the brains of TgNN6h, vCJD-infected mice, revealed the presence of florid plaques, which were restricted to the subcallosal area (x40). **(B)** vCJD-positive TgNN6h mice also showed large and dense PrP^{Sc} deposits in the vicinity of the corpus callosum. These deposits presented a multicentric morphology and were composed of amyloid since they stained positive with Congo Red (B, x40; insert picture x5). Immunohistochemistry was performed using the 3F4 antibody (1:1000).

Discussion

In the present study we aimed at assessing the way in which a wide array of prion isolates behaves when propagated in a human non-glycosylated model. Particularly, we were interested in finding whether the absence of glycans in the human PrP^C could impact the transmission barrier and the strain properties of BSE prions, given that BSE is the only animal prion strain for which a natural transmission to humans has been described, leading to its human counterpart vCJD, which maintains BSE pathobiological features (Bruce *et al.*, 1997; Hill *et al.*, 1997; Scott *et al.*, 1999). On PMCA, only BSE-related prions propagated in the TgNN6h substrate, suggesting the maintenance of the human transmission barrier for prions despite the lack of glycans of human PrP^C. vCJD strain propagated in the TgNN6h substrate showing the highest efficiency and, subsequently, it was the only prion strain able to transmit *in vivo* after the inoculation of the original isolate (Figure 2A). After 15 PMCA rounds, SSBP/1, atypical scrapie, CWD, L-BSE and H-BSE isolates could not be propagated in the human substrate (Figure 1). Although there are no reports demonstrating the natural transmission to humans of these prion diseases, multiple attempts to assess their zoonotic potential have been performed. Aside from classical BSE, the cross-species transmission of prions to humans has been demonstrated for classical scrapie (Cassard *et al.*, 2014), CWD (Barria *et al.*, 2014), and L-BSE (Beringue *et al.*, 2008a; Kong *et al.*, 2008; Comoy *et al.*, 2013) by the experimental challenge of transgenic mice overexpressing human PrP^C, and/or *in vitro* propagation techniques. However, these successful transmissions are the exception rather than the rule since many other similar studies have reported opposite results (Kong *et al.*, 2005; Sandberg *et al.*, 2010; Wadsworth *et al.*, 2013; Barria *et al.*, 2014), of course, considering that there is great variability in both the nature of the isolates and the human substrates used. Regarding our obtained results in the PMCA assay (Figure 1) and our preliminary results from the bioassay in TgNN6h and Tg340 mice (Table 1) we can suggest that the absence of glycans of human PrP^C has not altered the human transmission barrier for the strains used in the present study.

The implication of the glycosylation status of PrP^C on intra- and cross-species transmission of prion strains has been discussed at length, and several studies have suggested that glycosylation of PrP^C could be a key factor influencing the transmission barrier (DeArmond *et al.*, 1997; Priola and Lawson, 2001; Tuzi *et al.*, 2008; Wiseman *et al.*, 2015). Wiseman *et al.*, 2015 showed that transgenic mice expressing unglycosylated

murine PrP^C (G3 mice) were completely resistant to prion disease, which was associated to the absence of the first glycosylation site since the elimination of the second site of N-glycosylation resulted in a complete loss of the transmission barrier for human prions. However, they also suggested that the resistance shown by G3 mice could be attributed to other factors observed in this model, such as the lower PrP^C expression level, a different localization of the unglycosylated PrP^C, or the higher levels of C1-truncated PrP^C (Wiseman *et al.*, 2015). As aforementioned, we did not observe any significant alteration of the cross-species transmission barrier for prions in the TgNN6h non-glycosylated human substrate. Differences in the isolates and/or the transgenic model used could explain the discrepancies between our results and those obtained in the G3 transgenic line (Wiseman *et al.*, 2015). Nevertheless, it could also be possible that glycans affect prion propagation in a strain or species-dependent manner.

As a control of the *in vitro* assay, BSE-related inocula were also serially propagated in PMCA using wild-type, normally glycosylated human and bovine substrates (experiments performed in collaboration with Dr Joaquín Castilla and his team, CIC bioGUNE, Bizkaia, Spain). All BSE isolates readily propagated in the wild-type bovine substrate (first round of PMCA; data not shown). However, as observed with TgNN6h substrate, BSE isolates propagated in the human brain homogenate substrate with lesser efficiency, with the exception of vCJD, which propagated in the wild-type human substrate in the first round of PMCA. We can suggest that, in the present study, BSE retained its original strain characteristics regarding the results obtained with the C-BSE, sBSE and pBSE isolates. The propagation efficiency of these isolates was absolute in the bovine substrate, but they were less efficiently propagated in both the wild-type (100% of replicates in 3rd round, data not shown) and non-glycosylated human substrates (Figure 1A). BSE-derived prions, irrespective of their producing host (cattle, sheep, pig or human) transmit to transgenic mice expressing bovine PrP^C showing no differences in regard to survival times or pathobiological characteristics, indicating a lack of a transmission barrier for the propagation of BSE prions in a bovine PrP substrate (Torres *et al.*, 2014). However, BSE prions transmit poorly to transgenic human models (Asante *et al.*, 2002; Bishop *et al.*, 2006; Beringue *et al.*, 2008a; Torres *et al.*, 2014), indicating the existence of a strong transmission barrier between cattle and humans, even though the transmission of BSE to humans naturally occurs producing vCJD (Bruce *et al.*, 1997). In addition, we observed that both sBSE and pBSE isolates propagated in TgNN6h substrate

more efficiently than C-BSE (Figure 1). These results are in accordance with previous studies showing that experimental sBSE prions propagate more efficiently than cattle BSE in transgenic mice expressing human PrP^{C} (Padilla *et al.*, 2011; Plinston *et al.*, 2011), which was attributed to a better structural compatibility between sheep PrP^{Sc} and human PrP^{C} (Padilla *et al.*, 2011).

Although PMCA is not a quantitative method (Vidal *et al.*, 2015), our *in vitro* results suggest that pBSE converted the non-glycosylated human substrate more efficiently than the sBSE isolate (Figure 1). However, C-BSE, sBSE and pBSE inocula showed identical conversion efficiencies when propagated in a wild-type human substrate (round 3 of PMCA, data not shown). The pBSE isolate used in the present study was a brain homogenate obtained from pigs experimentally infected with the sheep BSE agent (Hedman *et al.*, 2016). Porcine PrP^{Sc} shows a predominance of the monoglycosylated fraction when cattle BSE (Seuberlich and Zurbriggen, 2010) or sheep BSE (Hedman *et al.*, 2016) are transmitted to pigs, unlike the rest of BSE-related strains, which are predominantly diglycosylated (Collinge *et al.*, 1996; Head *et al.*, 2004; Padilla *et al.*, 2011). In addition to the *in vitro* results obtained in TgNN6h substrate (Figure 1) we also observed that, although with a slight difference, the PMCA-propagated strain derived from pBSE (pBSE-PMCA) was the one that was most rapidly transmitted to TgNN6h mice among the BSE strains that were transmitted to these animals in a first passage (Table 1). Tuzi *et al*., (2008) showed that the strain 79A, which is a low glycosylated strain (Somerville *et al.*, 2005), was transmitted more efficiently to transgenic mice expressing unglycosylated PrP^{C} than other more highly glycosylated prion strains. Thus, they suggested that the different prion strains have specific requirements for each of the glycosylation sites of host PrP^{C} and, therefore, the TSE transmissibility could be affected by the compatibility between the glycosylation of host PrP^{C} and the glycoform ratio of the infecting strain (Tuzi *et al.*, 2008). Regarding the results obtained herein with the pBSE strain (Figure 1, Table 1), we could suggest that the characteristic glycoform ratio of this strain, predominantly monoglycosylated (Hedman *et al.*, 2016), might have slightly favored the propagation in a non-glycosylated substrate compared to the C-BSE and sBSE strains, which are predominantly diglycosylated (Padilla *et al.*, 2011).

However, we should consider that vCJD, also a predominantly diglycosylated strain (Head *et al.*, 2004), propagated in the TgNN6h non-glycosylated PrP^{C} more efficiently than any of the other BSE-related isolates used in the present study (Figure

1A, Table 1, Figure 2A). The higher efficiency of vCJD respect to C-BSE prions to propagate in wild-type human substrate has been previously reported (Barria *et al.*, 2014). Since PMCA was designed as a methodology to accelerate the misfolding process (Castilla *et al.*, 2005), we can suggest that the compatibility of amino acid sequences between seed and substrate and other slight adaptations of vCJD to the human brain environment could account for the high propagation efficiency in human substrate that we have observed for this given isolate, even considering that vCJD remains a BSE-related strain (Hill *et al.*, 1997). In addition, transmission studies in humanized transgenic mice and primates have demonstrated that BSE, although being a strain with very stable pathobiological features upon transmission, adapts to the new host (Lasmezas *et al.*, 2001; Bishop *et al.*, 2006), and once has transmitted to human beings in the form of vCJD, the transmission barrier for human-to-human is substantially reduced (Bishop *et al.*, 2006).This fact could also explain the results we have obtained in the TgNN6h mouse bioassay since vCJD was the only strain that transmitted to mice at first passage after the direct inoculation of the original isolate (Table 1, Figure 2).

To date, the bioassay in TgNN6h and Tg340 transgenic mouse models is still ongoing (Table 1). Nevertheless, all TgNN6h mice inoculated with the *in vitro* propagated BSEC-PMCA, sBSE-PMCA, or pBSE-PMCA strains succumbed to prion disease in ~200 dpi and proved positive for PrP^{res} accumulation by immunohistochemical techniques (Figure 4) and Western blot (Figure 2B). Animals inoculated with direct brain homogenates BSE, sBSE and pBSE did not develop disease within an equal or longer period of time. These results corroborate that the direct inoculation requires longer incubation times while PMCA serial propagation of these inocula in TgNN6h substrate greatly facilitates crossing the species barrier by the progressive stabilization and adaptation of the *in vitro* generated prions (Castilla *et al.*, 2008). BSE-PMCA, sBSE-PMCA, and pBSE-PMCA affected mice disclosed very severe neuropathological changes, showing abundant plaque deposits presenting the features of florid plaques described in vCJD-affected humans (Figure 4) (Will *et al.*, 1996). These three PMCA-propagated inocula were very similar with respect to the characteristics and distribution of the neuropathological features produced (Figures 3 and 4), which also coincide with those previously described for Tg340 (Padilla *et al.*, 2011) and Tg650 (Beringue *et al.*, 2008b) mice inoculated with BSE and BSE-related strains. These transgenic lines express a fully glycosylated human 129M PrP^{C} , and they also show florid plaques especially

affecting cerebral cortex, corpus callosum and thalamus when inoculated with BSE/vCJD prions (Beringue *et al.*, 2008b; Padilla *et al.*, 2011).

vCJD-positive TgNN6h mice also developed florid plaques, although they were scarcer than those observed with PMCA-adapted inocula (Figure 5A). In addition, the histopathological lesions were milder than those described in the brains of vCJD-infected Tg650 mice (Beringue *et al.*, 2008b). However, these differences are simply explained by the fact that the vCJD inoculum was not previously adapted by PMCA, and because the Tg650 transgenic line overexpresses human PrP^{C} at a 6-fold level (Beringue *et al.*, 2008b), making vCJD transmission more efficient at a first passage. Nevertheless, surprisingly, vCJD-positive TgNN6h mice also accumulated dense PrP-positive plaque-like deposits in the corpus callosum (Figure 5B). These large amyloid deposits, although located in the same brain area as the florid plaques found in these animals, showed a multicentric morphology. Thus, these neuropathological features cannot be classified as florid plaques since florid plaques are typically unicentric, arranged in amyloid radiating spicules (Ironside and Bell, 1997; Sikorska *et al.*, 2009). Very similar amyloid deposits were described in the proximity of the corpus callosum in 79A and ME7-inoculated transgenic mice that, curiously, also expressed a non-glycosylated PrP^{C} (Tuzi *et al.*, 2008). In this case, as in our study, it could be argued that these amyloid accumulations could be due to an aberrant localization of the non-glycosylated PrP^{Sc}, or to an innate tendency of these transgenic animals to form amyloid plaques. Dense plaque-like PrP^{Sc} deposits were also described in the corpus callosum of infected mice expressing a PrP devoid of the glycosylphosphatidylinositol (GPI) anchor (Chesebro *et al.*, 2005; Chesebro *et al.*, 2010). However, in this latter case, unlike that observed by Tuzi *et al*., 2008 and us in the present study, the most plausible explanation is that these extracellular amyloid plaques are caused by an aberrant localization of PrP^{Sc} aggregates since anchorless PrP in these mice is not attached to the plasma membrane, and this altered phenotype is seen with all the strains inoculated in the transgenic model (Chesebro *et al.*, 2005; Chesebro *et al.*, 2010; Mahal *et al.*, 2012). We only detected these large amyloid deposits in vCJD-infected mice, whereas age-matched TgNN6h mice inoculated with the rest of direct isolates did not show this type of PrP amyloid plaques (not shown), nor did the mice infected with the PMCA-propagated isolates. Therefore, we can suggest that these amyloid deposits appeared as a consequence of the inoculation with the vCJD direct isolate, rather than due to an effect of the lack of glycosylation, as previously proposed

(Tuzi *et al.*, 2008). It was also suggested that the aberrant formation of plaques could be a mechanism that the host adopts to protect itself from PrP^{Sc}, sequestering it as amyloid (Tuzi *et al.*, 2008) since it has been demonstrated that subfibrillar, small oligomers are significantly more pathological than large amyloid plaques (Caughey and Lansbury, 2003; Silveira *et al.*, 2005). This hypothesis was suggested as an explanation to the lack of clinical signs usually detected in prion-challenged mice that accumulate this type of aberrant amyloid plaques (Chesebro *et al.*, 2005; Piccardo *et al.*, 2007; Tuzi *et al.*, 2008). We also found these large amyloid deposits in vCJD inoculated mice that did not develop clinical disease, euthanized at >700dpi. However, we should not directly attribute the lack of clinical disease in these mice to the aberrant accumulation of amyloid. vCJD transmits poorly to transgenic mice overexpressing human PrP^{C} (Beringue *et al.*, 2008b; Padilla *et al.*, 2011; Torres *et al.*, 2014) so it is not surprising that TgNN6h mice, which express human PrP^{C} at ∼1 fold level (Haldiman *et al.*, 2013), do not develop clinical signs after inoculation of direct vCJD in a first passage. It is known that prion strains require an adaptation to the new host to stabilize their neuropathological hallmarks (Morales *et al.*, 2007). Thus, the large amyloid plaques observed in vCJD-infected TgNN6h mice could be simply explained because the strain is not completely adapted to the host and, therefore, the neuropathology at first passage has been more heterogeneous. If this is the case, it would be expected that in subsequent passages the strain will adapt to the transgenic model, reproducing the typical neuropathological hallmarks of vCJD/BSE prions. The analysis of the brains of vCJD-PMCA inoculated TgNN6h mice and/or a second passage of the direct isolate in TgNN6h mice (experiments still ongoing) will elucidate this question.

Overall, our results suggest that the neuropathological hallmarks of BSE were maintained after transmission to a humanized non-glycosylated host. When analyzed by Western blot, TgNN6h mice that developed disease displayed a single unglycosylated band with a wide range between 19-21 kDa with 3F4 (Figure 2B). This glycoprofile was almost identical to that displayed by *in vitro* propagated seeds in TgNN6h substrate, which showed an unglycosylated band at 19 kDa with 3F4 (Figure 1B) and absence or mild signal at 21 kDa with 12B2 antibody (experiments performed in collaboration with Dr Joaquín Castilla and his team, CIC bioGUNE, data not shown). These biochemical features coincide with those of classical BSE (Biacabe *et al.*, 2007). Thus, we can suggest that glycans are not necessary to maintain the pathobiological features of classical BSE

prions. Other studies have suggested that the glycosylation status of host PrP^C could strongly determine the phenotypic characteristics of the infecting strain (Cancellotti *et al.*, 2013) and the transmission efficiency of prions between different species (Wiseman *et al.*, 2015). However, these effects have been observed with some strains but not others, which led to the conclusion that glycans may not be essential to the retention of strain-specific properties (Cancellotti *et al.*, 2013),or that the strains present dramatically different requirements with respect to the glycosylation status of host PrP^C (Tuzi *et al.*, 2008). It has also been discussed that the interpretation of these results could be difficult since the mutations inserted to eliminate the glycosylation sites of PrP^C (DeArmond *et al.*, 1997; Neuendorf *et al.*, 2004; Tuzi *et al.*, 2008; Cancellotti *et al.*, 2013; Wiseman *et al.*, 2015) could be partly responsible for the observed alterations in the properties of some strains (Salamat *et al.*, 2011; Moudjou *et al.*, 2016). Accordingly with this suggestion, Piro *et al*., 2009 showed that unglycosylated PrP^{Sc} molecules, generated in vitro using an enzymatically deglycosylated mouse PrP^C as a substrate, maintained their strain-dependent neuropathological and biochemical features when inoculated in wild-type mice. These results led to the hypothesis that unglycosylated PrP^{Sc} molecules may be able to encode strain-specific patterns of PrP^{Sc} accumulation (Piro *et al.*, 2009). This conclusion was further supported by Moudjou *et al*., 2016 by the propagation of 126S scrapie prions in ovine PrP glycosylation mutants and their subsequent transmission to Tg338 ovinized mice. Non-glycosylated, PMCA-propagated 127S prions reproduced the neuropathological and biochemical features of normally glycosylated 127S prions when inoculated in Tg338 mice. In addition, these unglycosylated prions recovered the three-band pattern when propagated in wild-type PrP^C by PMCA. Thus, they concluded that glycans do not play a major role in determining strain-specific properties (Moudjou *et al.*, 2016).

As aforementioned, we observed that TgNN6h mice which developed the disease after transmission of BSE isolates, showed similar pathobiological features to those described in natural cases (Will *et al.*, 1996; Biacabe *et al.*, 2007) and in BSE/vCJD-challenged transgenic mice expressing a fully glycosylated human PrP^C (Beringue *et al.*, 2008b; Padilla *et al.*, 2011). The bioassay in TgNN6h and Tg340 mice is still ongoing and further *in vitro/in vivo* experiments would be necessary to test if the original glycosylation status of the BSE isolates is recovered after the propagation of the TgNN6h-PrP^{Sc} generated herein in a fully glycosylated substrate. However, overall, our results

suggest that glycans are not necessary to maintain neither the human transmission barrier for TSE nor the pathobiological features of BSE prions.

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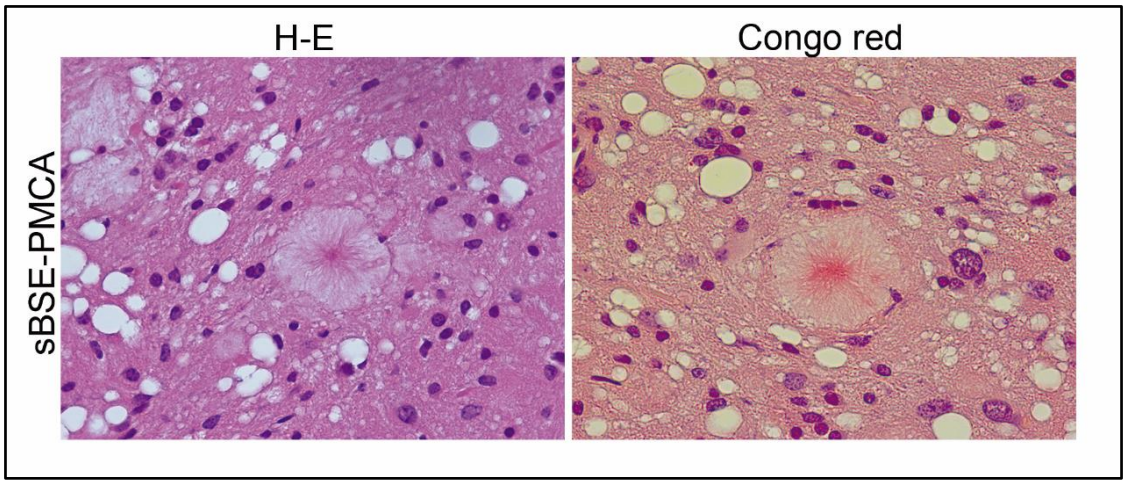
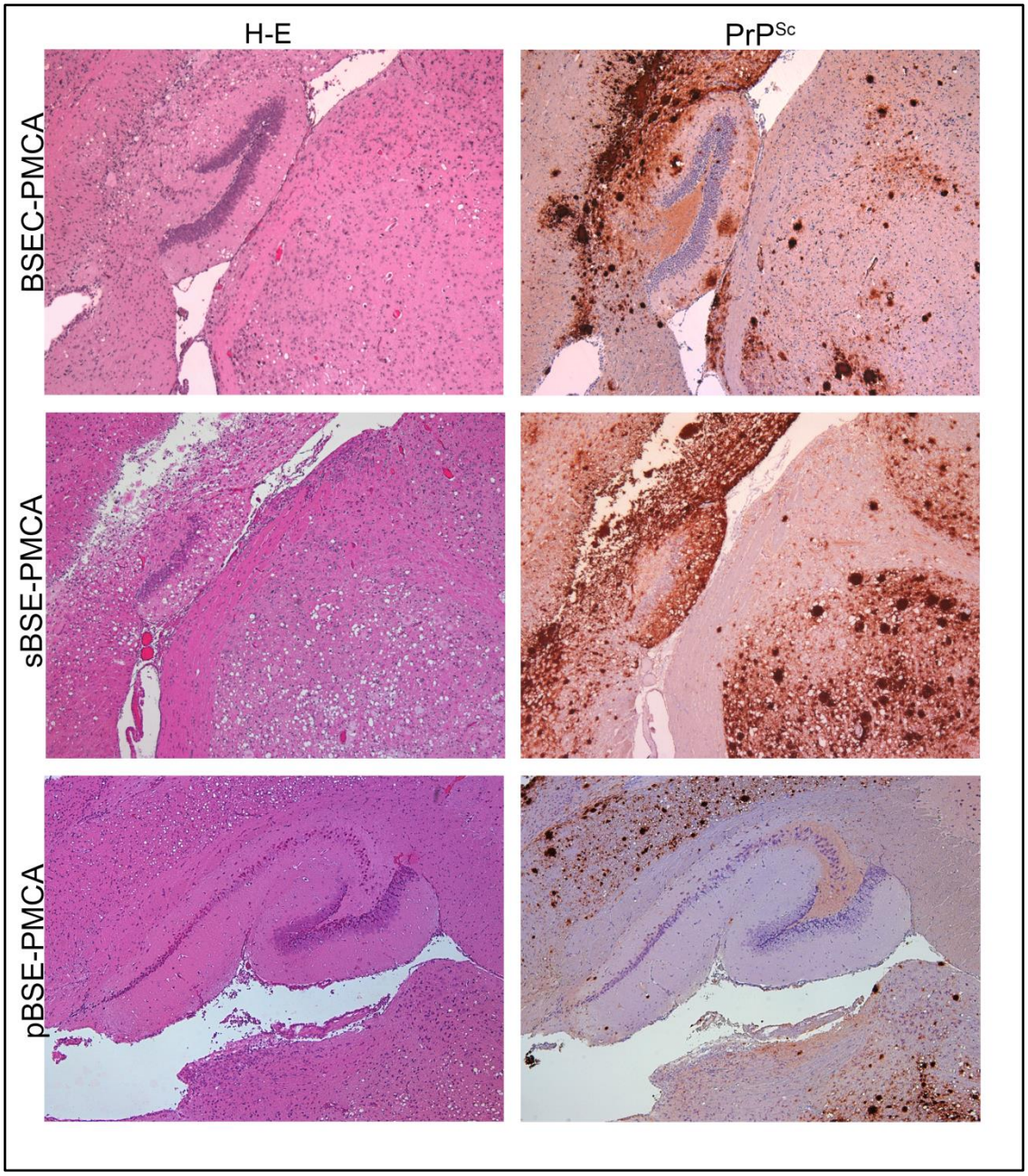
Supplementary material



Supplementary figure 1: Haematoxylin-eosin and Congo Red staining of a florid plaque observed in the thalamus of a sBSE-PMCA inoculated TgNN6h mouse (x40). The core and the radiating spicules of the florid plaques observed in TgNN6h mice were Congo Red positive, indicating an amyloid fibrils organization.



Supplementary figure 2: Spongiform changes and PrP^{Sc} deposits observed in the hippocampal area of TgNN6h mice inoculated with PMCA-propagated BSE isolates. Certain mice within each group presented very intense spongiform changes and PrP^{Sc} deposition in the subcallosal area and in the hippocampus, leading to tissue destruction and tissue loss (x5). Compare in the picture the animal inoculated with the sBSE-PMCA isolate with the mice inoculated with BSE-PMCA, or pBSE-PMCA isolates. This caused a great variability in the semiquantitative evaluation of this brain area.

ESTUDIO 4
Spontaneous prion-associated neurodegeneration in transgenic mice causes both ER stress and proteasome impairment at the terminal stage of prion diseases

Spontaneous prion-associated neurodegeneration in transgenic mice causes both ER stress and proteasome impairment at the terminal stage of prion diseases

Abstract

Prion diseases are a group of neurodegenerative disorders which can be sporadic, familial or acquired by infection. Conversion of the physiologically encoded prion protein PrP^{C} to its abnormal and misfolded isoform PrP^{Sc} is the main event in the pathogenesis of all forms of prion diseases. In sporadic forms, although several theories have been proposed, the mechanisms that trigger the appearance of PrP^{Sc} in the central nervous system, subsequently leading to neurodegeneration, remain unknown. Several reports have demonstrated that the accumulation of PrP^{Sc} can induce endoplasmic reticulum (ER) stress and proteasome impairment from early stages of the prion disease, and that both mechanisms lead to an increment of PrP aggregates in the secretory pathway, which could be of interest to understand the pathogenesis of sporadic prion diseases. Here, we investigate the role of ER stress and proteasome impairment during the course of sporadic prion disorders in a murine model of spontaneous prion disease (TgVole) coexpressing the Ub^{G76V}-GFP reporter, which allows measuring the proteasome activity *in vivo*. Sporadically prion-affected mice showed a significantly higher accumulation of both the foldase PDI and the Ub^{G76V}-GFP reporter than age-matched controls in certain brain areas. However, no significant ER stress or loss of proteasome function were detected in preclinical animals indicating that, contrarily to what has been observed in models of infectious prion diseases, neither of these two mechanisms appears to be an early event in the pathogenesis of sporadic prion diseases. Although further molecular and biochemical studies are necessary, our results show that, as previously reported, ER stress and proteasome impairment seem to be collateral events associated with prion neuropathology rather than essential mechanisms in the pathogenesis of sporadic prion diseases.

Introduction

Prion diseases or transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative disorders produced by the accumulation of the pathological prion protein (PrP^{Sc}) in the central nervous system (CNS) of affected individuals (Prusiner, 1998b). TSE can have a spontaneous, familial or infectious origin, but all prion-related disorders share a main pathogenic event: the conversion of the physiological cellular

prion protein (PrP^{C}) into its misfolded, abnormal isoform PrP^{Sc} (Prusiner, 1998b; Collinge, 2001).

Even though the pathogenic mechanisms of prion diseases are not completely known, it has been suggested that ER stress induced by PrP^{Sc} could play an important role in prion-associated neurodegeneration (Hetz and Glimcher, 2009; Torres *et al.*, 2010; Soto and Satani, 2011; Wang *et al.*, 2012). PrP^{Sc} accumulation has been reported to initially disturb ER calcium homeostasis (Hetz *et al.*, 2003b; Torres *et al.*, 2010) and to trigger the Unfolding Protein Response (UPR), a pro-survival mechanism which can be activated by the persistent accumulation of misfolded proteins in the ER producing ER stress (Malhotra and Kaufman, 2007; Kim *et al.*, 2008). The UPR results in the upregulation of ER chaperones and foldases, the decrease of protein synthesis and the induction of protein elimination through the ER-associated degradation (ERAD), a cellular pathway that involves the retro-translocation of misfolded proteins into the citosol and its degradation by the ubiquitin-proteasome system (UPS)(Hampton, 2000; Zhang and Kaufman, 2006; Kim *et al.*, 2008). The upregulation of certain ER chaperones, especially Grp78/Bip (a member of the glucose-related protein family) and protein disulfide isomerases such as PDIA1 (PDI) and PDIA3 (Grp58), has been demonstrated in prion diseases (Yoo *et al.*, 2002; Hetz *et al.*, 2005; Tang *et al.*, 2010; Wang *et al.*, 2012).

PDI is a folding enzyme present in the ER lumen at high concentrations (Lyles and Gilbert, 1991). This protein plays a key role in oxidative folding, catalyzing the formation and rearrangement of disulfide bonds in proteins (Freedman *et al.*, 1994; Wang *et al.*, 2015) but also acts as a molecular chaperone, promoting a correct folding and preventing aggregation of misfolded proteins (Freedman *et al.*, 1994; Wang, 1998; Wilson *et al.*, 1998; Bottomley *et al.*, 2001). The upregulation of proteins of the PDI family in the brain of prion affected individuals has been described in both variant Creutzfeldt Jakob disease (vCJD) and sporadic CJD (sCJD) patients (Yoo *et al.*, 2002; Hetz *et al.*, 2003b; Torres *et al.*, 2015) and in prion infected rodents (Hetz *et al.*, 2005; Wang *et al.*, 2012). The overexpression of these proteins was suggested to be a defense response against the presence of PrP^{Sc}, to try to correct the abnormal misfolding of the protein or increase its elimination (Yoo *et al.*, 2002; Hetz *et al.*, 2003b; Hetz *et al.*, 2005). *In vitro* studies using cell cultures demonstrated that PDI family member Grp58 interacts with PrP, acting as a neuroprotective factor (Hetz *et al.*, 2005). However, it was also

shown that PDI could have a pro-apoptotic effect in models of protein misfolding diseases leading to a cascade of caspases and apoptotic cell death (Hoffstrom *et al.*, 2010). Nevertheless, Wang *et al* (2012) demonstrated that the role of PDI during the course of prion disease is complex, exerting a protective activity against PrP^{Sc} at early stages, but inducing apoptosis at the terminal point of the disease.

As aforementioned, when the cell experiences ER stress, the UPR increases the expression of proteins of the PDI family (Turano *et al.*, 2002) and improves the protein elimination through the UPS (Zhang and Kaufman, 2006) Misfolded and unnecessary proteins to be eliminated via the UPS are tagged by multiple ubiquitin molecules and subsequently degraded by the 26S proteasome (Hershko and Ciechanover, 1998). However, it has been suggested that during the pathogenesis of prion diseases an impairment of this natural defensive mechanism may occur. Histopathological studies showed the accumulation of ubiquitinated aggregates in the brain in human prion diseases (Suenaga *et al.*, 1990; Ironside *et al.*, 1993). Subsequent experiments, using transgenic mice, have corroborated the aggregation of these ubiquitinated conjugates in the brain of prion infected animals (Kang *et al.*, 2004; Kristiansen *et al.*, 2007; McKinnon *et al.*, 2016) and *in vitro* studies have shown that PrP^{Sc} is able to inhibit the proteolytic β subunits of the proteasome decreasing its activity (Kristiansen *et al.*, 2007). All these results indicate that the deterioration of the UPS function may play an important role in the pathogenesis of neurodegeneration in prion diseases.

Both ER stress and UPS impairment, induced by PrP^{Sc}, can be correlated since the chronic ER stress leads to an accumulation of non-translocated PrP in the cytosol (Kang *et al.*, 2006; Orsi *et al.*, 2006) that could deteriorate the proteasome activity (Kristiansen *et al.*, 2007). *In vitro* and *in vivo* studies have shown that cells under ER stress fail to clear efficiently the aberrant proteins, which accumulate and cause an impairment of the UPS (Menendez-Benito *et al.*, 2005). It has been also demonstrated that both ER stress and proteasome activity inhibiton lead to an increment of insoluble PrP aggregates in the secretory pathway and cause a significant accumulation of PrP^{Sc} in persistently prion-infected cells suggesting that, therefore, these mechanisms could be involved in *de novo* formation of prions (Nunziante *et al.*, 2011) .

However, other studies failed to detect ER stress or proteasomal malfunction in transgenic models of familial prion diseases (Quaglio *et al.*, 2011). In the present study

we investigated the role of ER stress and UPS impairment in sporadic prion diseases. To this end, we studied the PDI accumulation and the deposition of the Ub^{G76V}-GFP reporter in Ub^{G76V}-GFP/1 mice, which allow monitoring the functionality of the UPS *in vivo* (Lindsten *et al.*, 2003). These mice also overexpressed bank vole I109 PrP, which leads to the development of a sporadic prion disease (Watts *et al.*, 2012). The accumulation of PDI and Ub^{G76V}-GFP may determine those cells undergoing ER stress and allows identifying which brain areas show evidence of UPS impairment. We also evaluated the co-localization of Ub^{G76V}-GFP aggregates and certain cell populations, with astrocytes being the cells with more intense accumulation. Our results show that both ER stress and UPS dysfunction are significantly incremented in certain brain areas, but only in the group of prion-clinical mice. Nevertheless, our study represents an approach, and further biochemical studies are necessary to enforce this hypothesis.

Materials and methods

Mouse brain samples

To study the accumulation and brain distribution of PDI and Ub^{G76V}-GFP over the course of spontaneous prion diseases we determined, by immunohistochemistry, the deposition of both proteins in paraffin-embedded brain samples from different transgenic mouse models. We selected mice expressing ~3-4x the I109 polymorphic variant of bank vole PrP (hereafter referred to as TgVole mice). The overexpression of bank vole PrP leads to the development of a spontaneous prion disease (Watts *et al.*, 2012) that, in this particular TgVole line, clinically appears at ~180 days of age. In addition, to study the impairment of the UPS in a model of sporadic prion diseases, double transgenic mice were used, generated by breeding TgVole mice with the Ub^{G76V}-GFP/1 mouse line (Lindsten *et al.*, 2003) (hereafter referred to as TgU1 mice). Thus, these mice also expressed the Ub^{G76V}-GFP reporter, which allows monitoring the UPS impairment since the accumulation of Ub^{G76V}-GFP indicates a deterioration of this system (Lindsten *et al.*, 2003). $TgU1^{+}$/$TgVole^{+}$ mice with a mean age of ~180 days represented the clinical group and the preclinical group consisted of $TgU1^{+}$/$TgVole^{+}$ mice with a mean age of ~60 days. Considering that aging produces oxidative stress which can decrease both the UPS activity and the functionality of ER chaperones such as PDI (Keller *et al.*, 2000; Brown and Naidoo, 2012), we selected two groups of healthy age-matched $TgU1^{+}$/$TgVole^{-}$ mice that were used as controls.

Immunohistochemical analyses

Sections from paraffin-embedded brains (4-µm thick) were cut and collected on glass slides and dried at 56ºC for 24h.

PDI, GFAP (glial fibrillary acidic protein), and PrP^{Sc} immunostaining were performed using an automated immunostaining system (Dako Autostainer) similarly to what has been previously described (Monleon *et al.*, 2004). After deparaffination and rehydration, sections intended for PrP^{Sc} immunostaining were pretreated with formic acid 98% and proteinase K (4 µg/ml; Roche, Switzerland) prior to hydrated autoclaving for 10 min at 121ºC while the samples used for PDI immunostaining were only subjected to the heat treatment. Next, endogenous peroxidase activity was blocked using a blocking reagent (Dako) and the samples were incubated for 1 h at room temperature with primary antibodies: anti-PrP 6H4 antibody (1:100; Prionics, Switzerland) or anti-PDI antibody (1:200; Santa Cruz Biotechnology, sc-166474). Sections were then incubated with an enzyme-conjugated anti-mouse Envision polymer (Dako) followed by diaminobenzidine (DAB, Dako), which was used as the chromogen.

GFP immunostaining was performed as described elsewhere (Kristiansen *et al.*, 2007), with few modifications. Briefly, sections were incubated with a peroxidase blocking reagent (Dako) for 30 min followed by 30 min with 10% goat serum in PBS (phosphate buffered saline). Immunodetection was performed overnight at 4ºC using a rabbit polyclonal anti-GFP primary antibody (1:2500; anti-GFP antibody-ChIP Grade, Abcam). The anti-rabbit Envision polymer (Dako) was used as the visualization system and DAB as the chromogen.

Brain sections were examined using a Zeiss Axioskop 40 optical microscope. PDI and GFP immunostaining were blindly evaluated in 9 encephalic areas: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellum (Cbl) and medulla oblongata (Mo)(Fraser and Dickinson, 1968) and semi-quantitatively scored on a scale of 0 (absence of immunolabeling) to 5 (very intense immunolabeling).

Dual immunofluorescence staining

Immunofluorescence staining of paraffin-embedded brain sections was performed as described previously (Sarasa *et al.*, 2012). Tissues were deparaffinated, rehydraded and subsequently blocked using 1% H2O2 for 30 min. Sections were then pretreated with 0,1% Triton X-100 for 3 h at room temperature and subjected to hydrated autoclaving (121ºC, 10 min). Immunodetection was performed overnight using primary antibodies: anti-GFP (1:200; Abcam) and anti-GFAP (1:200; Dako) which were diluted in a solution of 0.1% Triton X-100. Sections were then washed in cold PBS and incubated with secondary antibodies: goat anti-mouse IgG biotin conjugate (1:100; Invitrogen) and Alexa fluor 594 streptavidin conjugate (1:1000; Invitrogen) for 1 h in darkness. Finally, slides were washed and mounted in aqueous medium.

Sections were analyzed using a Zeiss fluorescence microscope Axioskop HBO (Carl Zeiss MicroImaging).

Data analysis

Differences in GFP and PDI immunostaining between groups of mice were evaluated using the non-parametric Mann-Whitney *U*-test and considered significant at $p < 0.05$. All data analyses and graphs were performed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

Results

PDI accumulation is increased in certain brain areas of spontaneously affected mice

Before analyzing the data, it was found that the expression of the Ub-GFP reporter had no apparent effect on the accumulation of PDI. Age-matched $TgU1^{+}/TgVole^{+}$ and $TgU1^{-}/TgVole^{+}$ mice showed an identical PDI accumulation pattern in terms of morphology of the deposits, intensity and brain distribution of PDI deposition. PDI immunostaining was more intense in mice that had developed the spontaneous prion disease in all brain areas. These animals showed a strong intraneuronal PDI immunolabeling, (Figure 1A, E) affecting numerous cells in certain brain regions. PDI positive immunostaining was found in all groups of mice, including healthy animals. However, the PDI accumulation in healthy mice was, in general, weaker and was detected in a smaller number of cells than in clinically affected mice. Dark-stained granular

aggregates were observed within the cytoplasm of neurons. Especially in the group of TgVole^{+} mice we also observed numerous strongly immunopositive cells whose morphology was consistent with glial cells (Figure 1A). Both the intensity of PDI labeling and the number of PDI positive cells were clearly lower in the Cbl of the preclinical TgVole^{+} mice (Figure 1B) and healthy adult TgVole^{-} mice (Figure 1C), which showed a similar PDI immunostaining. The number of PDI positive cells was especially low in the Cbl of healthy young TgVole^{-} mice (Figure 1D). This pattern of intracellular immunolabeling, characteristic of ER associated proteins (Hetz *et al.*, 2005; Torres *et al.*, 2015), was, in general, more evident in clusters of large neurons such as the deep nuclei of the cerebellum and the gigantocellular reticular nucleus of the medulla oblongata (Figure 1). No positive immunolabeling was found in the neuropil of any group of mice.

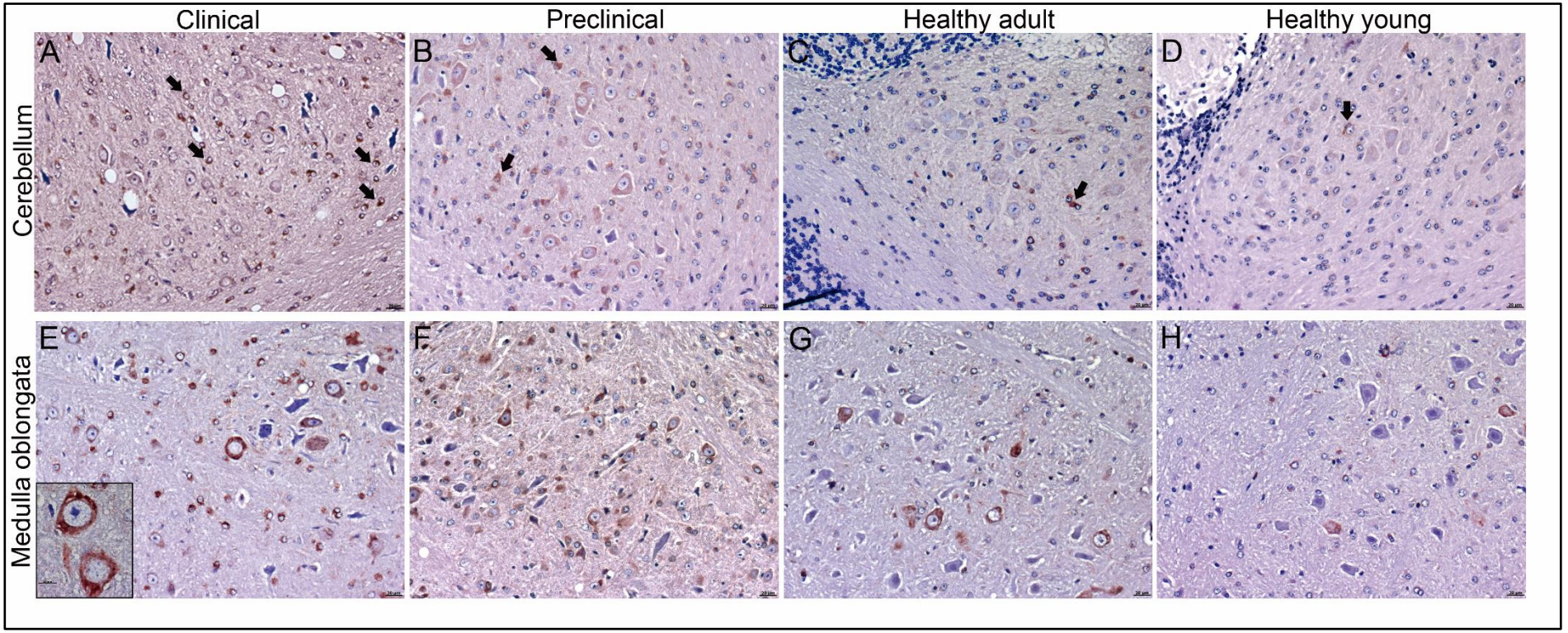


Figure 1: Immunodetection of PDI in cerebellum and medulla oblongata of spontaneously prion affected mice, preclinical mice and age-matched controls. **(A)** PDI immunostaining in the deep cerebellar nuclei of a clinical TgVole^{+} mouse, **(B)** a preclinical TgVole^{+} mouse, **(C)** a healthy adult TgVole^{-} mouse and **(D)** a healthy young TgVole^{-} mouse. All groups of animals showed immunopositive cells whose morphology was consistent with glial cells (arrows). **(E-H)** we also observed a strong intraneuronal PDI labeling in the gigantocellular reticular nucleus of the medulla oblongata. Insert picture in **(E)** contains two neurons showing a strong accumulation of PDI.

The semiquantification of the immunolabeling was statistically studied and a significant higher accumulation of PDI was demonstrated in the group of spontaneously sick animals in certain brain areas. Mo was the brain area presenting the most intense PDI accumulation in all groups of mice, regardless of age or genotype (Figure 2). Clinically affected mice presented statistically significant higher PDI scores in Fc, T, Ht, Cbl and Mo ($p<0.05$) than age-matched controls. Although preclinical mice showed a higher PDI deposition than healthy age-matched mice in all brain areas, no significant differences were obtained between both groups of animals (Figure 2A). However, PDI immunolabeling was significantly higher in Hc, T, Ht, Mes and Cbl of clinical mice than that observed in the same areas in the preclinical group. No significant differences were obtained between adult and young TgVole^{-} animals, indicating that aging does not produce a significant increase in PDI accumulation (Figure 2B).

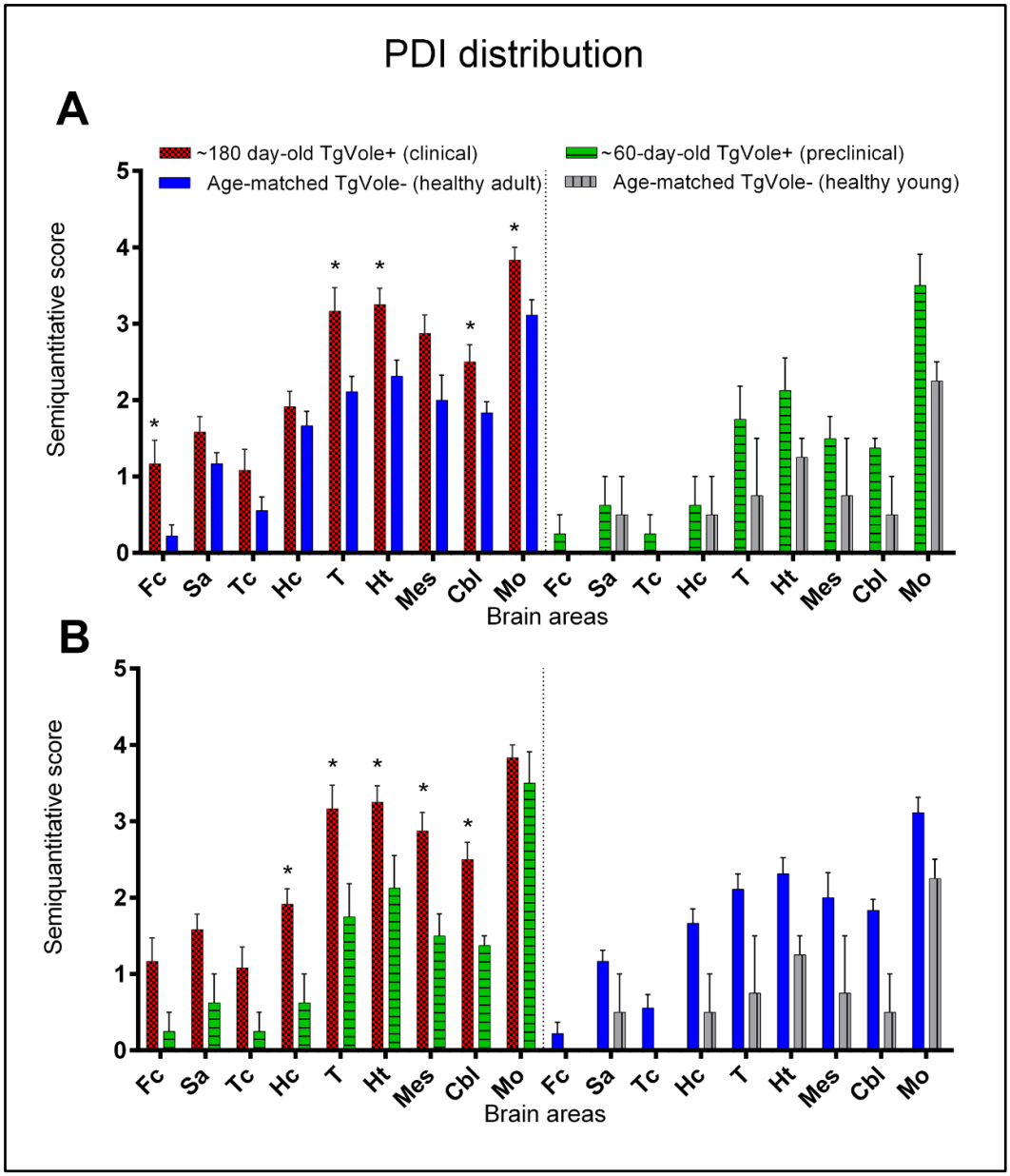


Figure 2: PDI distribution in the brains of clinical and preclinical TgVole$^+$ mice and their age-matched controls. PDI immunolabeling was semiquantitatively analyzed and scored on a scale of 0 (absence of immunolabeling) to 5 (very intense immunolabeling) in nine different brain areas: frontal cortex (Fc), septal area (Sa), thalamic cortex (Tc), hippocampus (Hc), thalamus (T), hypothalamus (Ht), mesencephalon (Mes), cerebellum (Cbl), and medulla oblongata (Mo). **A)** Comparison of the PDI immunolabeling profiles revealed significant differences between the group of TgVole$^+$ clinical animals and their age-matched controls in certain brain areas but not between preclinical TgVole$^+$ mice and their age-matched controls. **B)** Significant immunostaining differences were also obtained between clinical and preclinical TgVole$^+$ mice. (*p<0.05, Mann-Whitney *U* test).

Ub^{G76V}-GFP accumulates in brain areas with prion-associated neuropathology in spontaneously affected mice

Ub^{G76V}-GFP accumulation was very intense in the thalamus of both clinical and preclinical TgU1^{+}/TgVole^{+} mice (Figure 3A, E). These ubiquitinated deposits appeared as granular immunostaining in the neuropil and intense intracellular immunolabeling. Interestingly, Ub^{G76V}-GFP deposits were also observed in TgU1^{+}/TgVole^{-} mice, being more abundant in the group of adult animals in most of the brain areas studied (Figure 3C, D and Figure 4). However, scant intracellular immunostaining was observed in both groups of healthy mice. Granular immunolabeling and filamentous protein aggregates were the most abundant types of deposits observed in the neuropil of adult TgU1^{+}/TgVole^{-} mice (Figure 3C, F), whereas in the group of young TgU1^{+}/TgVole^{-} mice we observed punctiform neuropil and intraneuronal immunostaining (Figure 3D). The specificity of the Ub^{G76V}-GFP immunolabeling was manifested by the absence of staining in TgU^{-} /TgVole^{+} clinically affected mice (Figure 3G).

When age-matched groups of mice were compared, it was shown that Ub^{G76V}-GFP protein scores were higher in TgU1^{+}/TgVole^{+} clinical mice than those of healthy age-matched TgU1^{+}/TgVole^{-} mice in certain brain regions. Significantly increased Ub^{G76V}-GFP accumulation was observed in the Fc, Sa, Cbl, ($p<0.05$), T and Mo ($p<0.01$) of TgU1^{+}/TgVole^{+} mice that had developed a spontaneous prion disease, being the thalamus the most affected area (Figure 3A).

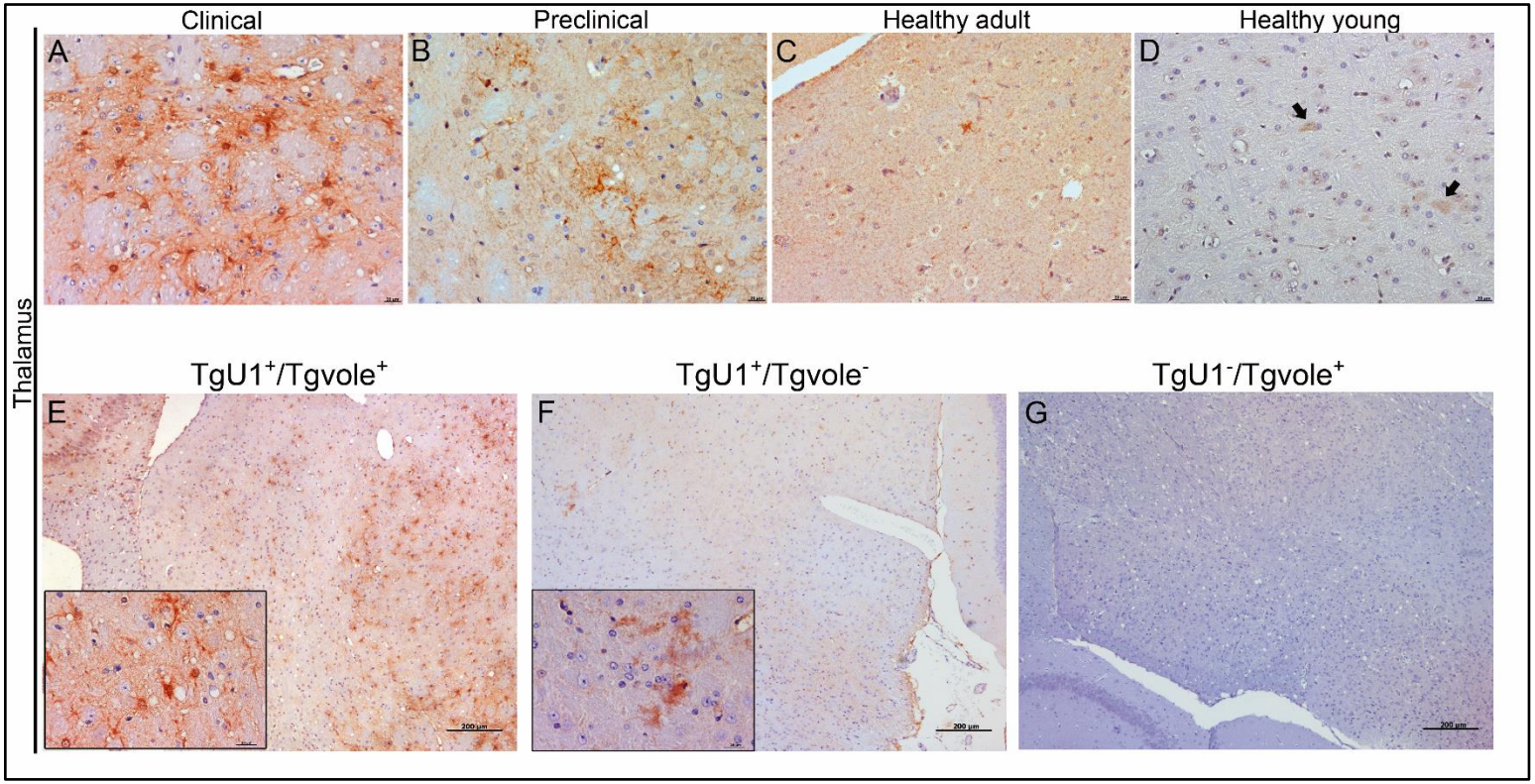


Figure 3: Ub^{G76V}-GFP accumulation in the thalamus of spontaneously affected mice, preclinical mice and age-matched controls. (A) Strong intracellular immunostaining in the thalamus of a TgU1^{+}/TgVole^{+} mouse that had developed the spontaneous prion disease associated with the overexpression of bank vole prion protein. **(B)** Collections of strong Ub-GFP positive cells were also observed in the thalamus of TgU1^{+}/TgVole^{+} preclinical mice, although Ub-GFP positive immunolabeling was observed in a lower proportion of cells than in clinical animals **(C)** Granular immunostaining and filamentous ubiquitin aggregates observed in the thalamus of a TgU1^{+}/TgVole^{-} healthy aged mouse. **(D)** Thalamus of a healthy young mouse showing slight intraneuronal Ub^{G76V}-GFP immunolabeling (arrows). Thalamic low-magnification images obtained from a **(E)** clinical and an **(F)** age-matched control to compare the extent of the ubiquitin deposition. Inserts contain magnified images of the corresponding sample to show the morphology of the deposits. **(G)** Absence of immunostaining in the thalamus of a clinical mouse not expressing the Ub^{G76V}-GFP reporter, indicating the specificity of the Ub^{G76V}-GFP immunolabeling. Note the intense spongiosis shown by this animal.

Thus, a certain degree of proteasomal dysfunction occurred as a result of aging, since all adult healthy mice presented ubiquitinated protein deposits in all analyzed brain areas. However, Ub^{G76V}-GFP accumulation was slightly incremented in clinical animals, suggesting that the spontaneous prion disease contributed to a higher dysfunction of the proteasomal degradation in certain regions of the CNS. This fact seems more evident in the thalamic area. In addition to the aforementioned statistical significant difference obtained in this area between clinical animals and age-matched controls, we observed that these two groups of mice, despite showing different immunolabeling intensity, present a similar Ub^{G76V}-GFP immunostaining distribution throughout the brain except in the thalamus, region in which a peak of Ub^{G76V}-GFP accumulation was observed in the group of clinical animals (Figure 4). When Ub^{G76V}-GFP scores of clinical and preclinical $TgU1^{+}/TgVole^{+}$ mice were compared, no significant differences were obtained, although clinical mice presented a higher accumulation of the GFP reporter in all brain areas. No differences were observed either between adult and young $TgU1^{+}/TgVole^{-}$ controls, although adults showed a more intense GFP immunolabeling in the brain. This fact indicates that, even though the increase in age seems to produce a certain level of Ub^{G76V}-GFP accumulation, this is not enough to cause a significant difference.

As aforementioned, we observed intracellular ubiquitinated deposition. The morphology of Ub^{G76V}-GFP immunopositive cells in certain brain areas, such as the hippocampus and thalamus, was very similar to that of reactive astrocytes. Considering that this immunolabeling pattern for Ub^{G76V}-GFP accumulation has already been described in RML-infected mice (McKinnon *et al.*, 2016), we performed an immunohistochemistry and a double immunofluorescence for GFAP (glial fibrillary acidic protein) and Ub^{G76V}-GFP in brain samples from spontaneously sick mice. GFAP staining revealed marked astrogliosis in certain brain areas. In these brain regions we also observed intense Ub^{G76V}-GFP immunolabeling affecting numerous cells whose morphology was consistent with hypertrophic astrocytes (Figure 5A). Dual immunofluorescence staining of Ub^{G76V}-GFP and GFAP confirmed that abundant GFP-positive cells were indeed reactive astrocytes (Figure 5B).

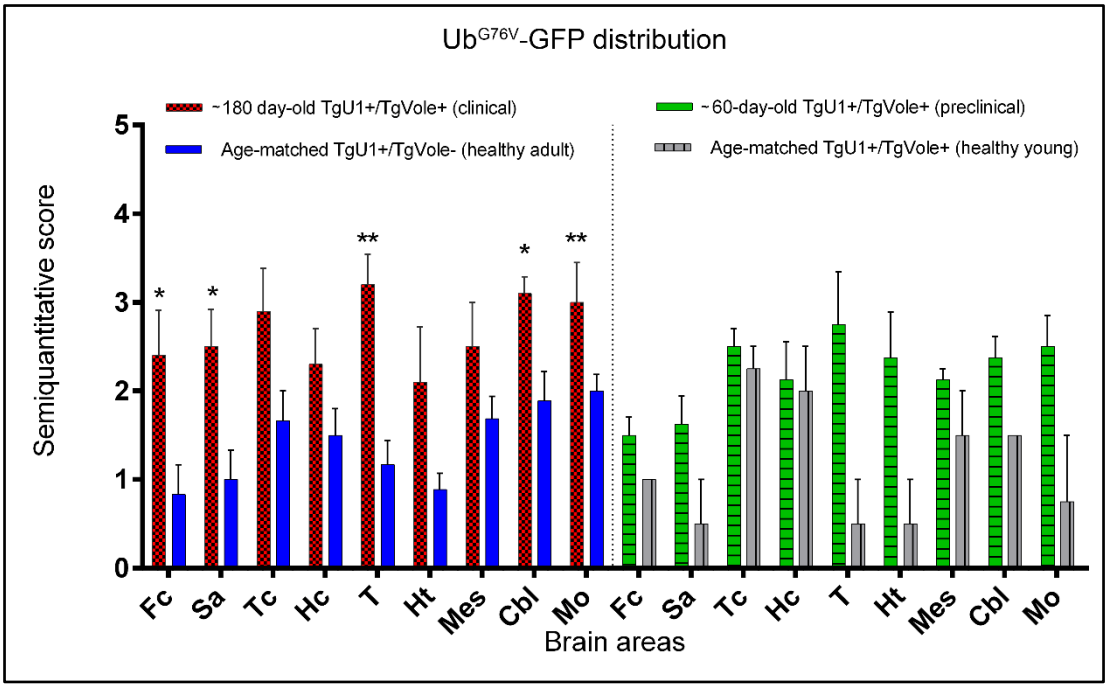


Figure 4: Ub^{G76V}-GFP distribution in the brains of clinical and preclinical TgVole^{+} mice and their age-matched controls Ub^{G76V}-GFP immunolabeling was semiquantitatively analyzed and scored on a scale of 0 (absence of immunolabeling) to 5 (very intense immunolabeling) in nine different brain areas. Comparison of the Ub^{G76V}-GFP immunolabeling profiles revealed significant differences between the group of TgU1^{+}/TgVole^{+} clinical animals and their age-matched controls in certain brain areas but not between preclinical TgU1^{+}/TgVole^{+} mice and their age-matched controls. No significant differences were obtained between clinical and preclinical TgU1^{+}/TgVole^{+} mice nor between adult and young TgU1^{+}/TgVole^{-} controls (graph not shown) (*p<0.05, Mann-Whitney *U* test).

Discussion

The exact molecular mechanisms involved in prion-associated neurodegeneration are, at present, mostly unexplained. The unknowns are even greater in the case of sporadic TSE since, although several theories have been suggested (Safar, 2012), their origin is still to be elucidated.

ER stress and UPS impairment have been proposed to play a pathogenic role in the neurodegenerative disorders associated with the accumulation of protein aggregates (Dantuma and Bott, 2014; Hetz and Mollereau, 2014; Scheper and Hoozemans, 2015). This is not unexpected since, when ER proteostasis is disturbed due to the accumulation of misfolded proteins, cells experience ER stress, and the UPR survival pathway is

initiated leading to the upregulation of ER chaperones and foldases (Zhang and Kaufman, 2006; Rutkowski *et al.*, 2008). In addition, the ER-associated degradation (ERAD) is improved, and unnecessary proteins are eliminated via the UPS (Hershko and Ciechanover, 1998; Brodsky and McCracken, 1999; Zhang and Kaufman, 2006). The accumulation of protein aggregates impairs the functionality of the UPS (Bence *et al.*, 2001) and thus, the malfunction of this system has been widely studied related to diseases produced by aberrant proteins (Ciechanover and Brundin, 2003; Dantuma and Bott, 2014). Moreover, it has been demonstrated that misfolded PrP inhibits the proteasome (Kristiansen *et al.*, 2007; Andre and Tabrizi, 2012) and that an impairment of the UPS occurs during the pathogenesis of acquired prion diseases (McKinnon *et al.*, 2016). Likewise, many other studies have shown an involvement of ER stress in infectious forms of TSE (Hetz *et al.*, 2003b; Hetz *et al.*, 2005; Torres *et al.*, 2010; Moreno *et al.*, 2013). However, regarding familial prion diseases, the roles of ER stress and UPS dysfunction are more controversial (Quaglio *et al.*, 2011; Wang *et al.*, 2011; Wang *et al.*, 2012).

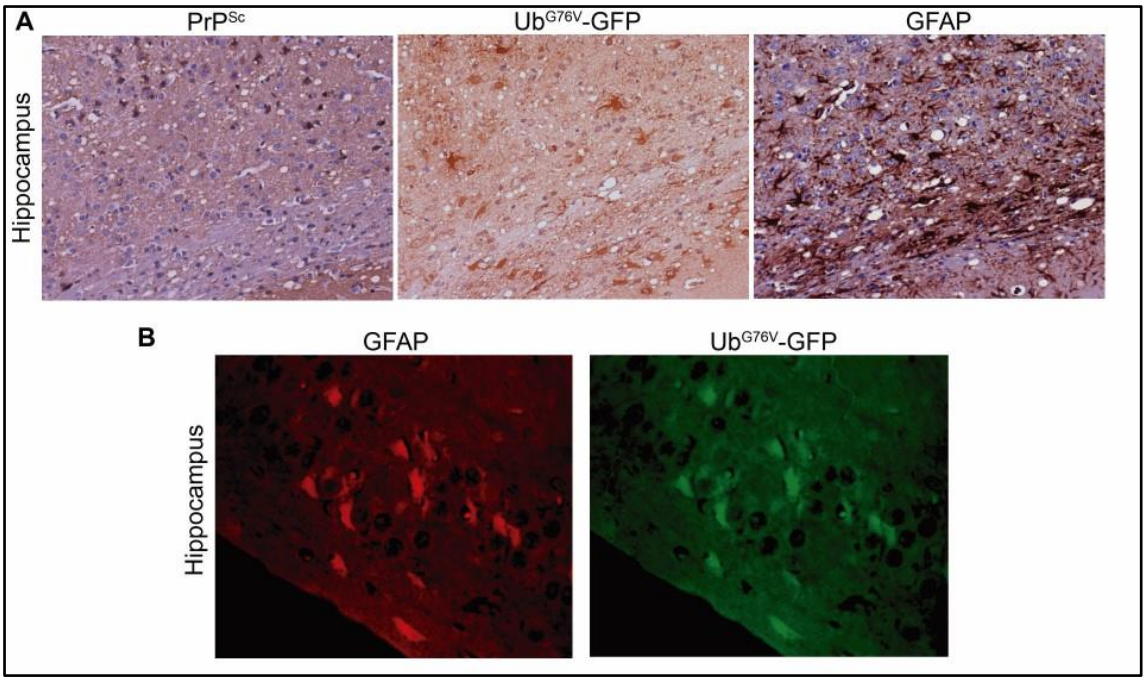


Figure 5: Ub^{G76V}-GFP intracellular accumulation is observed in brain areas showing prion-associated neuropathology. A) Hippocampus from a clinical TgU1^{+}/TgVole^{+} mouse stained for PrP^{Sc}, Ub^{G76V}-GFP and GFAP. Strong Ub^{G76V}-GFP immunolabeling is observed affecting numerous cells which appear to be reactive astrocytes. **B)** Dual immunofluorescence staining with ant-GFAP and anti-GFP antibodies revealed that numerous reactive astrocytes accumulated the Ub^{G76V}-GFP reporter.

In the present study we evaluated the possible participation of ER stress and UPS impairment in the pathogenesis of sporadic prion diseases. For this purpose, we analyzed the accumulation and brain distribution of PDI, a chaperone induced during ER stress (Perri *et al.*, 2015), and those of the Ub^{G76V}-GFP reporter, which allows to determine the functionality of the UPS *in vivo* (Lindsten *et al.*, 2003) in the brains of $TgU1^{+}/TgVole^{+}$ mice. Although an up-regulation of PDI and other disulfide isomerases such as Grp58/Erp57 has already been demonstrated in brain samples from sCJD patients (Yoo *et al.*, 2002; Hetz *et al.*, 2003b; Torres *et al.*, 2015), both ER stress and UPS impairment have not been deeply evaluated through the course of sporadic prion diseases. This fact is understandable since sequent time-course studies are more complicated to perform in the context of sporadic forms than in infectious TSE due to the difficulty of establishing exactly the precise moment in which the PrP^{Sc} appears in the CNS and, therefore, the moment in which the neuropathogenic mechanisms begin. Thus, we selected brain samples obtained from $TgU1^{+}/TgVole^{+}$ mice culled at different ages in order to evaluate these pathogenic events in the preclinical and clinical stages of the spontaneous prion disease. The overexpression of the bank vole I109 PrP leads to the development of spontaneous neurodegeneration in TgVole mice, making them a suitable model for the study of the sporadic forms of TSE (Watts *et al.*, 2012).

We performed an immunohistochemical approach to evaluate the accumulation of the ER stress marker PDI in the brains of clinical and preclinical TgVole mice. Despite the fact that clinical mice presented a greater accumulation of PDI in all evaluated brain areas, we observed PDI positive immunolabeling in all groups of animals (Figure 2), regardless of age or genotype. In addition, although dynamic assays of PDI expression during scrapie experimental infection have demonstrated that the upregulation of this protein begins at an early stage and continues to increase till terminal stage (Wang *et al.*, 2012), this does not seem to occur in our case. Preclinical $TgVole^{+}$ mice showed a significant lower accumulation of PDI than clinical $TgVole^{+}$ in certain brain regions (Figre 2B), which could lead us to think that during the course of spontaneous TSE there is also a sequential increase of this enzyme, however it is necessary to point out that healthy adult mice show a higher PDI immunostaining than preclinical mice in almost all brain areas evaluated, therefore indicating that at least in the preclinical stage there is no correlation between prion pathogenesis and PDI overexpression. Clinical $TgVole^{+}$ mice presented a greater accumulation of PDI in T, Ht and Cbl both with respect to healthy

adults and preclinical mice (Figure 2). Thus, we can suggest that prion replication in sporadic prion diseases might be accompanied by an overexpression of PDI in certain brain areas, and that this phenomenon could merely represent a neuronal response against the accumulation of PrP^{Sc} since it seems to occur in the clinical stage of the disease. Similar conclusions were obtained in previous studies in which an overexpression of Grp58, the closest homologue of PDI (Koivunen *et al.*, 1996), was found in the terminal phase of CJD-affected humans and murine scrapie models (Yoo *et al.*, 2002; Hetz *et al.*, 2003b). However, later it was demonstrated that a significant upregulation of Grp58 can be already detected during the preclinical phase of murine scrapie (Hetz *et al.*, 2005), an event that does not seem to occur in our case. Therefore, although we found some significant differences between sporadically prion-affected and healthy mice, ER stress does not seem to be an essential pathway during the course of the sporadic neurodegenerative disorder developed by TgVole mice. Other studies have also shown that, although ER stress is apparently induced in prion diseases, the genetic ablation of proteins directly involved in ER stress response or in ER-stress mediated apoptosis does not alter the progression of the disease *in vivo* (Steele *et al.*, 2007; Hetz *et al.*, 2008), suggesting, as in our case, that this cellular mechanism might not be a major pathogenic event during the course of prion diseases. However, further biochemical and molecular analyses are necessary to corroborate the role of PDI in sporadic prion diseases.

We also investigated the possible pathogenic role of proteasome impairment at different stages of the sporadic prion disease in TgVole mice. In a similar way to what was observed for PDI, GFP immunoreactivity was detected in the brains of all mice expressing the Ub^{G76V}-GFP reporter. However, in this case, the differences between the group of clinical and age-matched controls were more evident in certain brain areas such as T and Mo (Figure 4). Interestingly, we observed that, especially in the thalamic area, the morphology of the Ub^{G76V}-GFP reporter deposits was very different between animals expressing the bank vole PrP (i.e. preclinical and clinical $TgU1^{+}/TgVole^{+}$ mice) and healthy $TgU1^{+}/TgVole^{-}$ mice. A high proportion of the cells in which the Ub^{G76V}-GFP reporter was detected in $TgU1^{+}/TgVole^{+}$ mice appeared to be reactive astrocytes (Figure 5A), which was later confirmed by dual immunofluorescence staining for GFAP and GFP (Figure 5B). An intense accumulation of the Ub^{G76V}-GFP reporter has already been described in the reactive astrocytes of the thalamus of RML-infected mice , in which a proportional increase in the number of GFP-labeled astrocytes was observed as

astrogliosis develops, and therefore, as the prion disease progresses (McKinnon *et al.*, 2016). These results agree with those we have observed in the present study, since we also found abundant GFP positive astrocytes in certain brain areas of the preclinical mice, and the proportion of these cells was clearly increased in the same brain areas of clinical $TgU1^{+}/TgVole^{+}$ animals. By contrast, Ub^{G76V}-GFP deposits found in healthy mice were morphologically different. In $TgU1^{+}/TgVole^{-}$ animals we detected filamentous and granular GFP-labeled aggregates in the neuropil, but we did not observe the astrocytic pattern found in $TgU1^{+}/TgVole^{+}$ mice (Figure 3). In addition to the aforementioned Ub^{G76V}-GFP accumulation in reactive astrocytes described in RML-infected mice (McKinnon *et al.*, 2016), it has been shown in other diseases that GFAP accumulation causes a decrease of proteasome activity (Tang *et al.*, 2006) and that misfolded proteins produce an astrocytic upregulation of UPS proteins and proteasome impairment (Lopez Salon *et al.*, 2003). We have also observed an accumulation of the Ub^{G76V}-GFP reporter in reactive astrocytes, suggesting that in sporadic prion diseases this phenomenon also occurs. However, the differences in the accumulation of Ub^{G76V}-GFP have only been observed between the group of clinical $TgU1^{+}/TgVole^{+}$ mice and their age-matched $TgU1^{+}/TgVole^{-}$ controls, and only in certain brain areas (Figure 4). Hence, we can suggest that in the present study, as previously described in models of genetic prion diseases (Quaglio *et al.*, 2011), UPS impairment seems to be a secondary event associated with the development of prion-associated neuropathology and then is only detected in advanced stages of the $TgVole^{+}$ spontaneous neurodegenerative disorder.

Therefore, our results do not clearly show a major role of ER stress or UPS dysfunction in the pathogenesis of spontaneous prion diseases. Although certain *in vivo* studies have positively demonstrated that both upregulation of PDI and UPS impairment are mediators of prion pathogenesis during the course of infectious TSE (Kristiansen *et al.*, 2007; Wang *et al.*, 2012; McKinnon *et al.*, 2016) others have failed to demonstrate a significant contribution of ER stress or proteasome malfunction in prion pathology (Unterberger *et al.*, 2006; Quaglio *et al.*, 2011). We cannot know for certain if during the spontaneous TSE of TgVole mice the ER stress and the UPS impairment are mere secondary events associated with prion neuropathology, as previously suggested (Yoo *et al.*, 2002; Quaglio *et al.*, 2011), or whether the PrP^{Sc} generated in our case, as has also been suggested, do not experience an ER metabolism but a Golgi-based quality control (Ashok and Hegde, 2009; Wang *et al.*, 2012) and therefore it does not activate the UPR

and, consequently, the mechanisms of ER-associated degradation. Nevertheless, as already mentioned, further molecular and biochemical studies are necessary to confirm the participation of ER stress and proteasome malfunction in the pathogenesis of sporadic prion diseases. Likewise, the role of other mechanisms of proteostasis regulation, as well as the role of other ER resident chaperones involved in folding quality control should be also explored in relation to the pathogenesis of these diseases.

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. ESTUDIO 5
Resistance-associated deer prion protein polymorphisms limit peripheral PrP^{CWD} deposition

Resistance-associated deer prion protein polymorphisms limit peripheral PrP^{CWD} deposition.

Abstract

Chronic wasting disease (CWD) is a prion disease affecting members of *Cervidae* family. PrP^{C} primary structures play a key role in CWD susceptibility resulting in extended incubation periods and regulating the propagation of CWD strains. Resistance-associated polymorphisms limit the tropism and distribution of PrP aggregates in the brain and peripheral tissues. We analyzed the distribution of abnormal prion protein (PrP^{CWD}) aggregates in peripheral organs from orally inoculated white-tailed deer expressing four different *PRNP* genotypes: Q95G96/Q95G96 (wt/wt), S96/wt, H95/wt and H95/S96. Although the different genotypes showed a similar PrP^{CWD} deposition patterns in the brain, we found that deer expressing the H95 PrP^{C}, despite having the longest survival periods, accumulated a remarkably lower amount of PrP^{CWD} deposits in peripheral organs. In addition, no PrP^{CWD} aggregates were detected in skeletal muscles of any of the deer genotypes. Our data suggests that, in orally infected deer, expression of H95-PrP^{C} limits peripheral accumulation of PrP^{CWD} as detected by immunohistochemistry. Conversely, infected S96/wt and wt/wt deer presented with similar PrP^{CWD} peripheral distribution at terminal stage of disease, suggesting the S96-PrP^{C} allele, although delaying CWD progression, does not completely limit the peripheral accumulation of the infectious agent.

Introduction

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) of cervids, the only known TSE found in both farmed and free-ranging animals (Williams and Young, 1980, 1982; Spraker *et al.*, 1997). Like other TSE, CWD is a fatal neurodegenerative disease caused by the conversion of the host-encoded cellular prion protein (PrP^{C}) to a misfolded isoform (PrP^{CWD}) through unknown mechanisms. CWD affects cervid populations in North America, South Korea and, between 2016-2018, it was reported for the first time in Europe, in a free-ranging reindeer (Benestad *et al.*, 2016), moose and red deer in Southern Norway and Finland. Horizontal transmission by direct animal interactions and via persistence of infectivity in the environment hinders control and eradication of these diseases (Miller *et al.*, 2004; Johnson *et al.*, 2006b; Schramm *et al.*, 2006; Almberg *et al.*, 2011).

It has been widely documented that certain polymorphisms of the prion protein gene (*PRNP*), encoding PrP^{C}, play a key role in the susceptibility to TSE (Dickinson *et al.*, 1968; Westaway *et al.*, 1987; Goldmann *et al.*, 1990; Palmer *et al.*, 1991; Bossers *et al.*, 1996). The close relationship between variability at *PRNP* and the susceptibility to CWD in cervids has been also demonstrated (O'Rourke *et al.*, 1999; O'Rourke *et al.*, 2004; Johnson *et al.*, 2006a). In white-tailed deer, the prevalence of CWD is lower in deer expressing at least one copy of the H95 or S96 polymorphisms (Johnson *et al.*, 2006a). The direct effect of these polymorphisms on disease progression was evaluated through experimental oral infection studies where the CWD source, the inoculated dose and the route of infection were controlled. This experimental infection demonstrated that the H95 and S96 polymorphisms impact CWD progression since deer homozygous for the wild-type (wt) alleles (Q95/G96) presented shorter incubation periods and a more rapid clinical disease period than deer expressing at least one copy of H95 and S96 alleles (Johnson *et al.*, 2011). It was further demonstrated that the H95 allele, in addition to greatly increasing the survival period of deer orally challenged with wt-CWD prions (Johnson *et al.*, 2011), modulated the emergence of the novel prion strain $H95^{+}$, which possesses singular biochemical and biological properties (Duque Velasquez *et al.*, 2015; Herbst *et al.*, 2017) .

Given the effects of PrP^{C} polymorphisms in CWD progression and pathogenesis, they might also produce an effect on prion accumulation. PrP^{C} primary structure influences the distribution of abnormal PrP aggregates (Ligios *et al.*, 2004; Spiropoulos *et al.*, 2007). Together with the infecting strain, the *PRNP* genotype is a major factor influencing the neuropathological phenotype (Bruce *et al.*, 1991; Parchi *et al.*, 1996; Gonzalez *et al.*, 2002; Spiropoulos *et al.*, 2007; Gonzalez *et al.*, 2012; Moore *et al.*, 2016).

Although less studied, variability at *PRNP* may affect the pathways of neuroinvasion and the involvement of other tissues (Gonzalez *et al.*, 2014; Hoover *et al.*, 2017). In sheep scrapie, the expression of arginine at position 171 has profound repercussions on PrP^{Sc} replication and distribution (Goldmann *et al.*, 1990; Goldmann *et al.*, 1994; Westaway *et al.*, 1994; Belt *et al.*, 1995; Clouscard *et al.*, 1995). R171 heterozygous sheep show lower accumulation of PrP^{Sc} in the lymphoreticular system (LRS) and other tissues as compared to Q171 homozygous sheep (van Keulen *et al.*, 1996; Andreoletti *et al.*, 2000; Gonzalez *et al.*, 2014). In CWD, it has been observed that PrP^{CWD} deposition in the brain and other organs progress at a slower rate in deer expressing

polymorphisms associated with a reduced susceptibility (Fox *et al.*, 2006; Johnson *et al.*, 2006a; Hoover *et al.*, 2017). However, observations are often made in free-ranging, naturally infected animals, which limit the conclusions that can be obtained about the potential effect of the genotype on PrP^{CWD} deposition, due to the variability in the infecting strains, routes of exposure and incubation periods.

Using immunohistochemistry (IHC), we evaluated PrP^{CWD} deposition in orally inoculated white-tailed deer expressing different *PRNP* genotypes: wt/wt, S96/wt, H95/wt and H95/S96 (Johnson *et al.*, 2011). Our study presents a thorough neuropathological characterization of the PrP^{CWD} distribution in white-tailed deer. We observed that deer expressing the H95 PrP^{C} accumulated less PrP^{CWD} in peripheral organs, especially in tissues related to excreta production.

Materials and Methods

Animals and tissue sampling

The brain and a set of peripheral tissues were collected from CWD orally inoculated white-tailed deer expressing different *PRNP* genotypes: Q95G96/Q95G96 (wt/wt; N=5; D1-D5), S96/wt (N=3; D6-D8), H95/wt (N=1; D9) and H95/S96 (N=1; D10) (Johnson *et al.*, 2011), using the Wisc-1 wt/wt CWD isolate (Johnson *et al.*, 2011; Duque Velasquez *et al.*, 2015). All deer had developed terminal clinical prion disease, showing different survival periods depending on their PrP^{C} primary structure (Johnson *et al.*, 2011). Corresponding samples from non-infected white-tailed deer of wt/wt *PRNP* genotype (N=2; D11 and D12) were also collected and examined as controls.

PrP^{CWD} immunohistochemical analysis

Collected samples were fixed in 10% formalin and embedded in paraffin. Tissues were cut into 5-μm-thick sections and mounted on glass slides for immunohistochemical analysis. PrP^{CWD} immunolabeling was performed using the monoclonal antibody (mAb) 6H4, followed by incubation with a secondary anti-mouse antibody, a peroxidase-streptavidin conjugate, a substrate chromogen and hematoxylin counterstain, as previously described (Johnson *et al.*, 2011). Sections were then scanned using a Hamamatsu NanoZoomer 2.0RS digital scanner (Hamamatsu Photonics, Hamamatsu, Japan).

The distribution, morphology and intensity of PrP^{CWD} deposits were blindly evaluated in 15 brain areas: Obex, cerebellar molecular layer (Cml), cerebellar Purkinje cell layer (Cpl), cerebellar granular layer (Cgl), cerebellar white matter (Cwm), Pons, superior colliculus (SC), thalamus (TH), hypothalamus (HT), caudate nucleus (CA), septal nucleus (SN), hippocampus (HC), frontal cortex grey matter (FCgm), frontal cortex white matter (FCwm) and olfactory bulb (OB), similarly to that previously described (Fox *et al.*, 2006). The intensity of PrP^{CWD} accumulation in each brain area was semi-quantitatively scored on a scale of 0 (absence of deposits) to 4 (severe deposition) in order to obtain a PrP^{CWD} brain profile for each genotype. GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA) was used to graph the neuropathology results.

The presence of PrP^{CWD} deposits was also evaluated in the following tissues: lymph nodes (retropharyngeal, submandibular, axillary, prescapular, prefemoral, popliteal, inguinal, tracheobronchial, ileocecal, hepatic, pancreatic and adrenal), spleen, third eyelid, tonsil, pituitary gland, peripheral nerves (vagus, brachial plexus and sciatic), skeletal muscle, heart, intestine, liver, pancreas, kidney, adrenal glands, lung, salivary glands (parotid, submandibular and sublingual), retina and optic nerve. PrP^{CWD} immunostaining in these tissues was subjectively scored as: - (absence of immunostaining), + (minimal to slight immunostaining), ++ (moderate immunostaining) and +++ (strong and extensive immunostaining).

Results

PrP^{CWD} deposition in lymphoid tissues and nervous system

PrP^{CWD} deposition was detected by immunohistochemistry in lymphoid tissues and the brain from all clinically affected deer regardless of *PRNP* genotype. PrP^{CWD} deposits appeared as bright-red granular material in Peyer´s Patches, tonsils, spleen and lymph nodes from CWD-challenged deer. In general, PrP^{CWD} immunolabeling was more intense in the lymph nodes of the head and visceral lymph nodes, whereas lymph nodes of the limbs (prescapular, axillary, prefemoral, popliteal and inguinal) showed a lower number of positive follicles and milder immunostaining in all deer. Although consistent with these observations, one S96/wt animal (D8) showed no PrP^{CWD} deposition in axillary, prescapular, prefemoral and inguinal lymph nodes despite its long incubation period (Johnson *et al.*, 2011). Lymphoid follicles of third eyelid and rectal mucosa were

strongly PrP^{CWD} positive when the histological sample contained follicles that allowed the immunohistochemical analysis (Table 1).

Brain samples presented intense PrP^{CWD} immunolabeling in all deer genotypes. The PrP^{CWD} profile was characterized by plaques and coarse granular and coalescing extracellular deposits mainly located around neurons, glial cells, vacuoles and along myelinated axons of the white matter. Although less frequent, intraneuronal PrP^{CWD} deposition was also observed, especially in the dorsal motor nucleus of the vagus nerve, the hypoglossal nucleus, the spinal trigeminal nucleus and the inferior olivary nucleus of the obex of all clinical deer.

Surprisingly, each *PRNP* genotype presented a distinguishable PrP^{CWD} pathological phenotype in the cerebellum. Wt/wt deer showed severe PrP^{CWD} immunostaining in granular layer with coarse granular and large plaques invading the Purkinje cell layer and extending to the molecular layer (Figure 1A). PrP^{CWD} plaques were also present in the cerebellum of all S96/wt clinically affected deer. However, for animals of this genotype, the presence of plaques was restricted to granular layer and white matter, whereas the Purkinje cell and the molecular layer showed milder granular and diffuse PrP^{CWD} deposits compared to wt/wt deer (Figure 1B). Conversely, the cerebellar pathological phenotype of the H95/wt deer was characterized by discontinuous and diffuse PrP^{CWD} labeling in the granular layer, showing predominantly fine punctate and coarse small granular deposits (Figure 1C), although a few plaque-like deposits were also observed. Finally, the cerebellum of the H95/S96 deer showed fine punctate and coarse granular PrP^{CWD} deposits homogeneously distributed through the granular layer. The cerebellar molecular layer of this deer presented a more intense immunolabeling than deer of other genotypes, showing conspicuous stellate PrP^{CWD} aggregates (Figure 1D).

In the frontal cortex, the morphological PrP^{CWD} profile of the H95/S96 deer differed from that observed in the other deer genotypes. All wt/wt, S96/wt and the H95/wt deer presented abundant coalescing deposits and large PrP^{CWD} plaques in both grey and white matter (Figure 1E). H95/S96 deer, however, had milder staining in the frontal cortex, presenting coarse and diffuse granular, cell-associated aggregates mostly confined to grey matter (Figure 1F), whereas white matter deposits were sparse, and predominantly of the perivascular type.

Table 1: Distribution of PrP^{CWD} deposits, detected by IHC, in lymphoid tissues of clinically affected and non-inoculated white-tailed deer.

											Non-inoculated	
Genotype	wt/wt					S96/wt			H95/wt	H95/S96	wt/wt	
	D1	D2	D3	D4[a]	D5	D6	D7	D8	D9	D10	D11	D12
Retropharyngeal LN	++	+++	+++		++	++	++	+++	++	+++	-	-
Submandibular LN	+++	++	++		+++	++	++	++	/	/	-	-
Axillary LN	++	++	+		+++	++	++	-	++	+	-	-
Prescapular LN	+	+	+		++	+	-	-	+	++	-	-
Prefemoral LN	++	+	++		+	+	+	-	+	++	-	-
Popliteal LN	+	+	++		+++	+	+	+	+	+	-	-
Inguinal LN	+	+	++	/	+++	+	+	-	/	++	-	-
Tracheobronchial LN	++	+	+++		+++	/	/	/	/	/	-	-
Ileocecal LN	/	++	++		+++	++	+++	++	++	+++	-	-
Hepatic LN	/	+++	/		/	++	++	+++	+++	+++	/	/
Pancreatic LN	+++	/	/		/	++	+++	/	/	/	-	/
Adrenal LN	+++	++	/		-	++	+++	++	/	++	/	-
Spleen	+	+	+	+	+	-	+	+	+	+	-	-
3rd eyelid	+	+	++	/	-[b]	-[b]	-[b]	+	/	+	-	-
Tonsil	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-
Peyer´s Patches	+++	+++	+++	+++	+++	+++	+++	/	+++	+++	-	-
Rectal lymphoid follicles	+++	+++	+++	+++	++	+++	+	/	/	+++	-	-

[a] No lymph nodes were collected from D4 [b] No lymphoid follicles were present in the histological sample

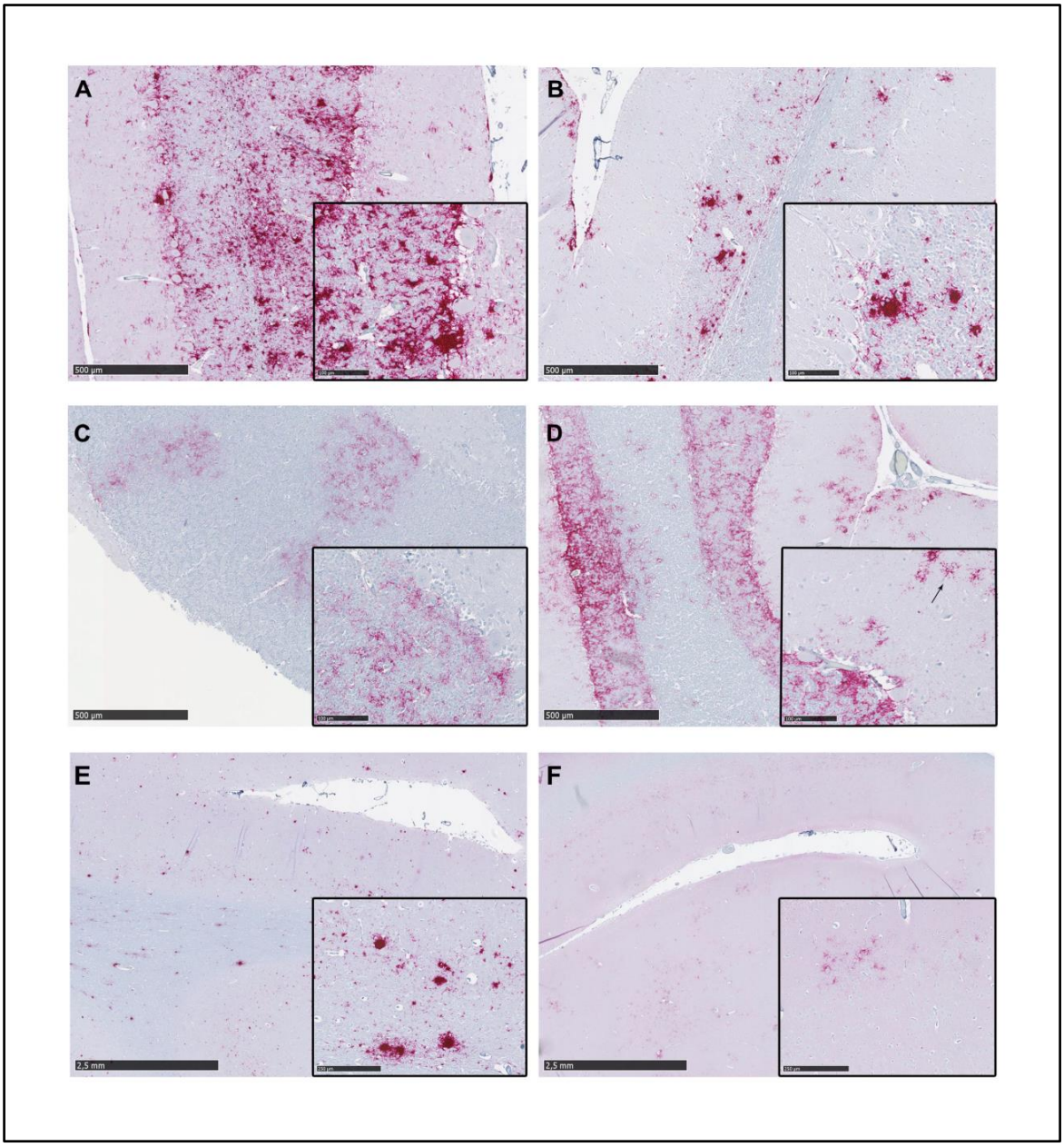

Figure 1: PrP^{CWD} deposition pattern in the cerebellum (A to D) and the frontal cortex (E, F) of white-tailed deer of different *PRNP* genotypes infected with CWD. Inserts contain magnified images of the corresponding sample to show the morphology and limits of the PrP^{CWD} aggregates. **(A)** Cerebellum of a wt/wt deer showing abundant coalescing PrP^{CWD} deposits and plaques in the granular and Purkinje cell layer. **(B)** S96/wt deer showing evident milder deposition. PrP^{CWD} plaques are observed only in the granular layer. **(C)** Cerebellum from the H95/wt deer showing a patch-shaped distribution of coarse granular and fine punctate PrP^{CWD} aggregates through the granular layer. **(D)** Cerebellum from the H95/S96 deer, which presented coarse granular aggregates homogeneously distributed through the granular layer and stellate aggregates in the molecular layer (arrow). **(E)** Frontal cortex from a wt/wt deer showing abundant plaques in grey and white matter. **(F)** Frontal cortex from the H95/S96 deer showing mild deposition of fine punctate cell-associate aggregates.

PrP^{CWD} neuroanatomical distribution was similar for all deer irrespective of their *PRNP* genotypes. Intense staining was observed in obex, superior colliculus, hypothalamus, septal nucleus of the basal ganglia and cerebellar granular layer in all deer (Figure 2E). Some differences however, were observed between these deer. Compared to deer of other genotypes, H95/S96 deer showed remarkably lower PrP^{CWD} accumulation in thalamus, frontal cortex and olfactory bulb (Figure 2). This lower accumulation may explain the different PrP^{CWD} morphological features found in frontal cortex (Figure 1F).

Optic nerves were positive for IHC accumulation in all clinically affected deer. In the rest of peripheral nerves, PrP deposition was irregular. The vagus nerve was the most consistent, with chromagen granules found in 5 of the 6 evaluated samples. However, only a few aggregates were detected in 2 of the 8 sciatic nerves evaluated and the brachial plexus was negative for PrP^{CWD} accumulation in all deer (Table 2).

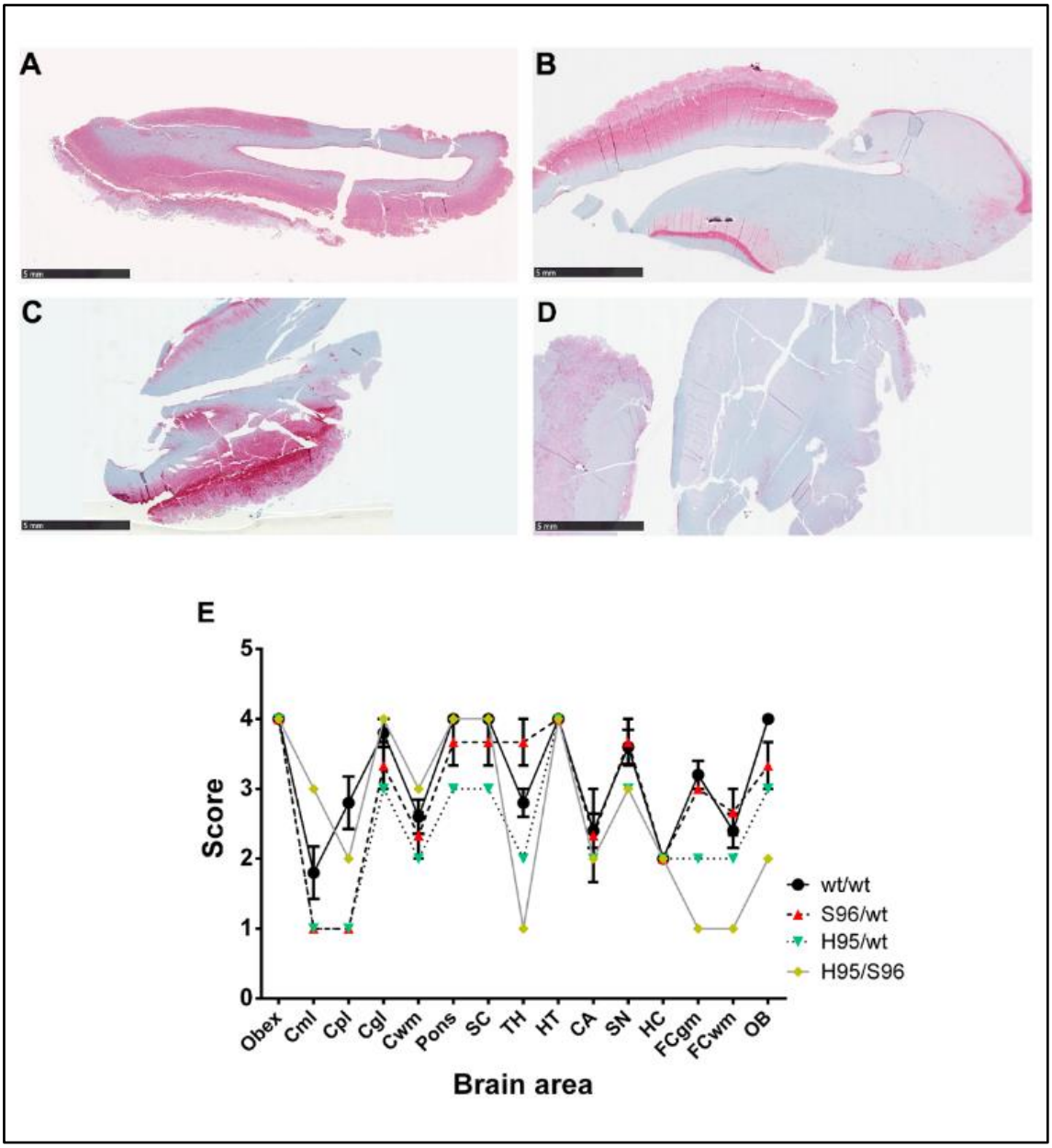

Figure 2: PrP^{CWD} deposition in the brain of CWD infected deer. (A) Representative olfactory bulb sample from a wt/wt deer showing abundant PrP^{CWD} immunolabeling diffusely distributed in the brain area. **(B)** Olfactory bulb sample from a S96/wt and the **(C)** H95/wt deer showing similar PrP^{CWD} immunolabeling, more restricted to the grey matter **(D)** Olfactory bulb sample from the H95/S96 deer showing mild PrP^{CWD} immunolabeling. **(E)** PrP^{CWD} deposition profile of the experimentally infected deer of different *PRNP* genotypes. Evaluated brain areas are: Obex; Cml, cerebellar molecular layer; Cpl, cerebellar Purkinje cell layer; Cgl, cerebellar granular layer; Cwm, cerebellar white matter; Pons; SC, superior colliculus; TH, thalamus; HT, hypothalamus; CA, caudate nucleus; SN, septal nucleus; HC, hippocampus; FCgm, frontal cortex grey matter; FCwm, frontal cortex white matter; OB, Olfactory bulb.

Table 2: Distribution of PrP^{CWD} deposits, detected by IHC, in peripheral nerves, glands and peripheral organs of clinically affected and non-inoculated white-tailed deer.

Genotype	wt/wt					S96/wt			H95/wt	H95/S96	Non-inoculated wt/wt	
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
Optic nerve	++	+	++	+	+	+	/	+	+	++	/	-
Vagus nerve	++	+	+	/	/	+	-	+	-	+	-	-
Brachial plexus	/	-	-	/	/	-	-	/	/	-	-	-
Sciatic nerve	+	+	-	/	-	-	/	-	-	-	-	-
Pituitary gland	/	++	+++	/	/	+++	+++	+++	/	+++	-	-
Islets of Langerhans	++	++	++	++	++	++	+	+	-	-	/	-
Adrenal Gl medulla	+++	+++	+++	+++	++	+++	+++	+++	+	++	/	-
Ileoc. Valve villi/crypts	++	+	+	+	++	++	++	/	-	-	-	-
Kidney	++[b]	+	+	+	+	/	+	+	-	-	-	-
Parotid salivary gland	+	+	-	/	+	+	-	+	-	/	/	-
Submandibular sal. Gl	/	+	-	/	+	+	+	+	-	-	/	-
Sublingual sal. Gl	/	-	-	/	/	-	-	-	-	-	/	-
Retina	+++	+++	+++	+	+++	+++	+	+++	++	++	/	-
Skeletal muscle	-	-	-	-	-	-	-	-	-	-	-	-
Heart	/	/	/	/	/	++	/	/	-	-	/	/
Lung	+[b]	-	-	-	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-	-	-	-	-

[b]Deer showing inflammatory kidney disease and lung interstitial inflammation

PrP^{CWD} deposition in endocrine tissues

Pituitary gland was collected from six clinically affected deer, two wt/wt, three S96/wt and the H95/S96 deer. This gland presented abundant PrP^{CWD} deposition in all deer, especially affecting pars nervosa and pars intermedia, although PrP^{CWD} staining was also present in pars distalis. In addition, PrP^{CWD} immunolabeling was observed in the pancreases of clinically affected wt/wt and S96/wt deer, restricted in the islets of Langerhans (Table 2 and Figure 3A, B) which are scattered clusters of endocrine cells. In these animals, positive islets of Langerhans were abundant and often adjacent, as previously described for CWD (Sigurdson *et al.*, 2001). Interestingly, no PrP^{CWD} deposits were observed in pancreatic tissues of either the H95/wt or H95/S96 deer (Table 2 and Figure 3C, D).

Adrenal glands were also positive by IHC in all clinically affected genotypes. Although most abundant immunolabeling was detected in the adrenal medulla, positive immunolabeling was also detected in the adrenal cortex. The presence of immunopositive material in the adrenal cortex was more abundant in the group of wt/wt deer.

PrP^{CWD} deposition in muscular tissues

Skeletal muscle samples were collected from tongue; forelimb and hindlimb muscles of the CWD challenged deer. No PrP^{CWD} immunolabeling was detected within skeletal muscle tissues. PrP^{CWD} deposits were neither observed in muscle-associated nerve fascicles, structures that have been previously reported to show PrP^{CWD} accumulation in CWD infected white-tailed deer (Daus *et al.*, 2011). However, it was observed that heart samples collected from a S96/wt deer (D6), showed clearly positive immunolabeling. In this S96/wt deer we observed scattered PrP^{CWD} aggregates, affecting separated groups of myocytes (Figure 4A). In addition, PrP^{CWD} immunolabeling was more visible in longitudinal cross-sections of the cardiac muscle, an accumulation pattern similar to that previously described in the heart of white-tailed deer infected with CWD (Jewell *et al.*, 2006). Surprisingly, all cardiac muscle samples from H95/wt and H95/S96 deer were negative for IHC deposition (Figure 4B, C).

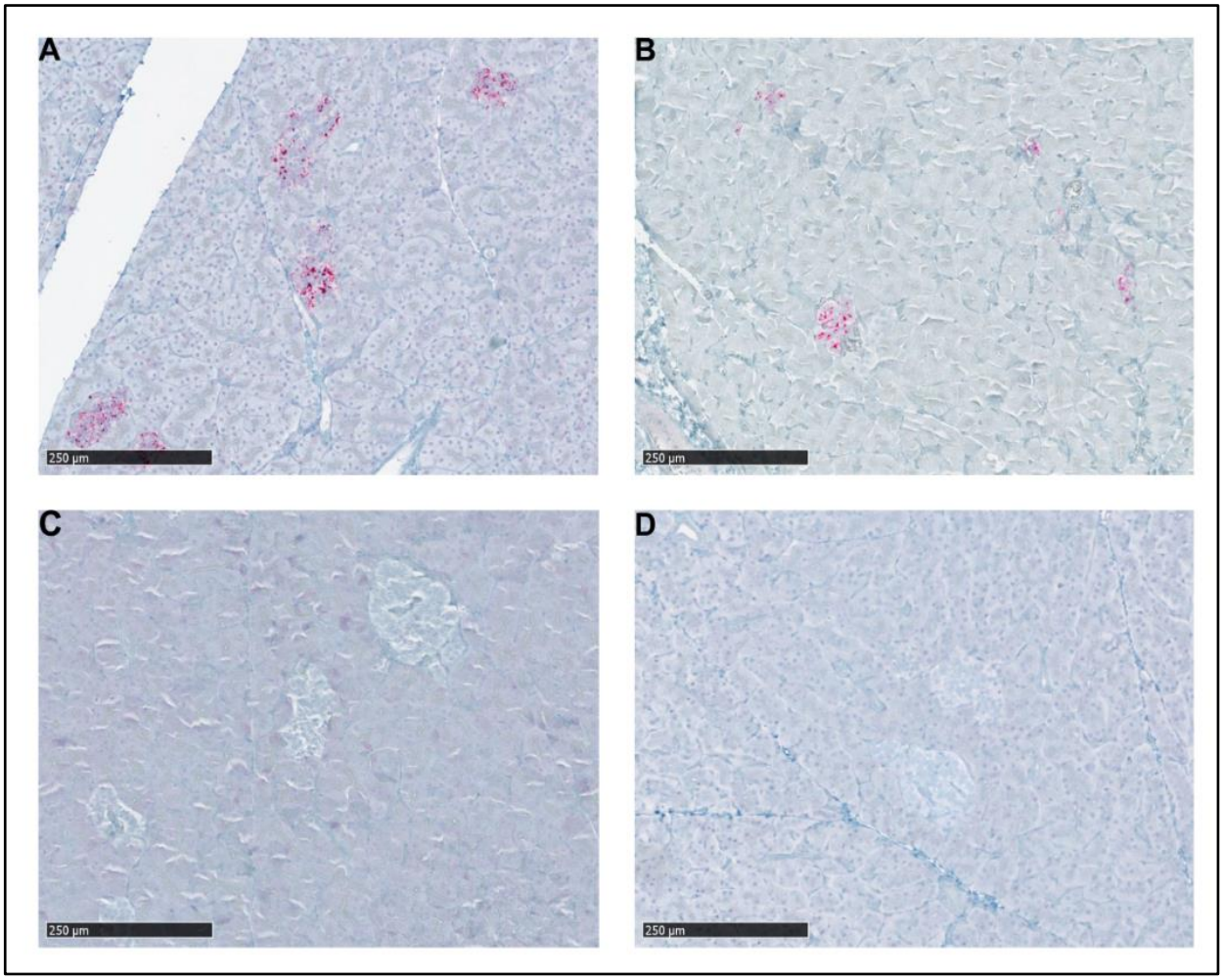

Figure 3: Immunohistochemical detection of PrP^{CWD} in the pancreas of white-tailed deer of different *PRNP* genotypes infected with CWD. (A) Pancreas from a wt/wt deer and a **(B)** S96/wt deer showing PrP^{CWD} deposition in the islets of Langerhans. **(C)** Pancreas from the H95/wt and the (**D)** H95/S96 deer, which did not present any positive immunolabeling.

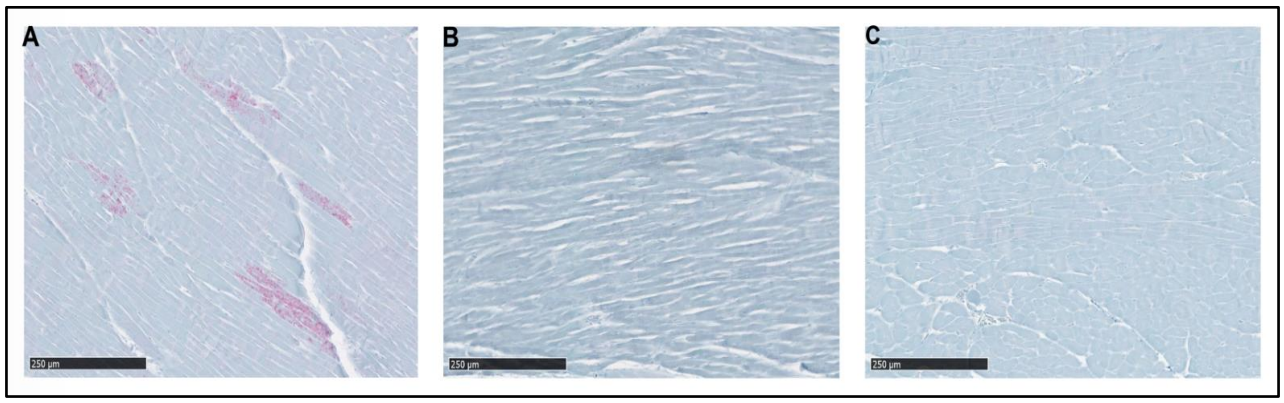

Figure 4: Immunohistochemical detection of PrP^{CWD} in the heart of white-tailed deer of different *PRNP* genotypes infected with CWD. (A) Heart from a S96/wt deer showing positive immunolabeling in separated groups of myocytes **(B)** Heart from the H95/wt and the **C)** H95/S96 deer in which no positive immunolabeling was detected.

PrP^{CWD} deposition in intestinal tract

Gut-associated lymphoid and nervous tissues accumulated high levels of PrP^{CWD} in all groups of clinical deer. IHC positive material was more abundant in Peyer´s Patches and nerve fibers and ganglia of the Enteric Nervous System (ENS) in all intestinal segments evaluated. However, differences in the distribution of PrP^{CWD} in the intestinal tract were evident between genotypes. The H95/wt and H95/S96 deer presented reduced accumulation of PrP^{CWD} in the villi and crypts of the intestinal mucosa compared to wt/wt and S96/wt deer. Differences were most noticeable at the ileocecal junction, with all wt/wt and S96/wt deer showing strong PrP^{CWD} deposition dispersed along the lamina propria between villi and crypts, while H95/wt and H95/S96 deer showed no immunolabeling (Figure 5).

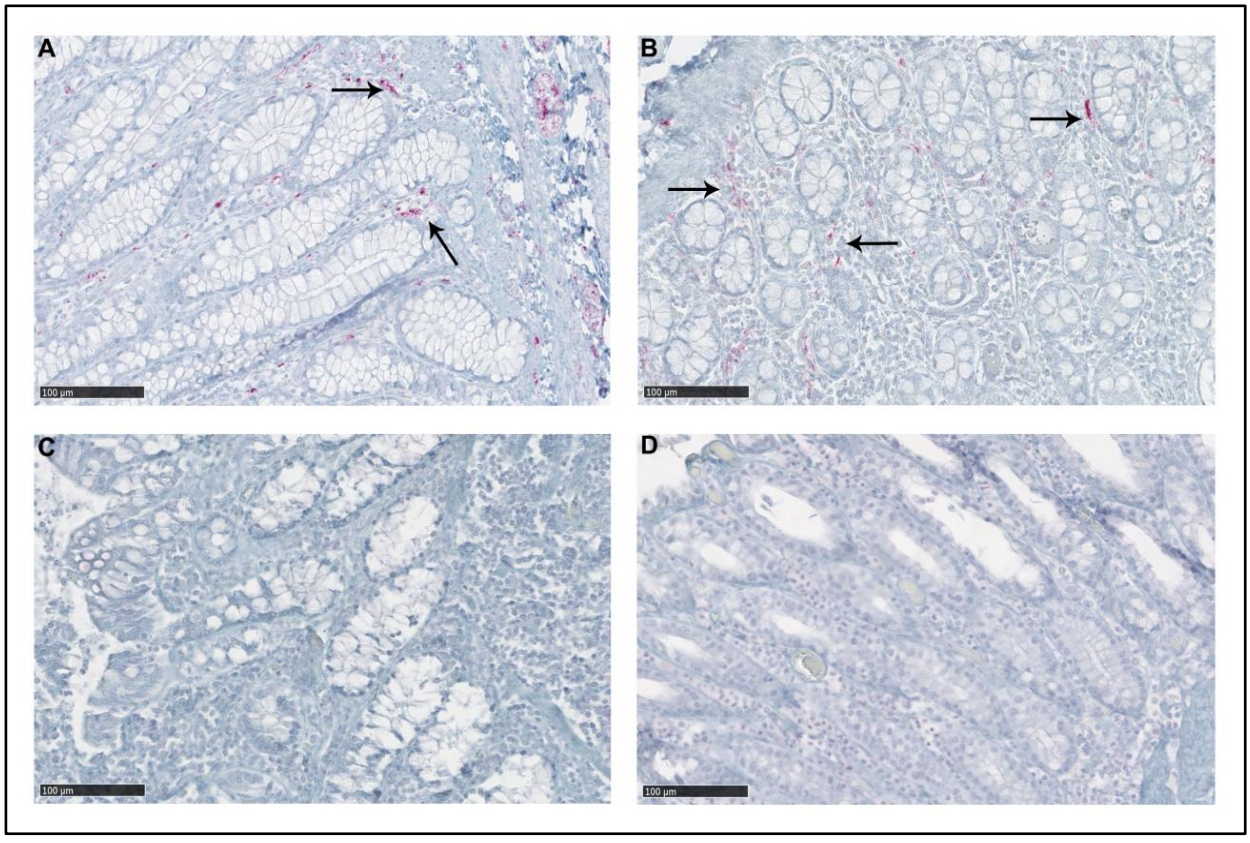

Figure 5: PrP^{CWD} deposition detected by IHC in the crypts of the ileocecal junction of white-tailed deer of different *PRNP* genotypes infected with CWD. (A) Ileocecal junction mucosa from a wt/wt deer and a **(B)** S96/wt deer showing PrP^{CWD} immunolabeling dispersed along the lamina propria between intestinal crypts (arrows). This PrP^{CWD} immunolabeling pattern was not observed in the ileocecal junction of the (**C)** H95/wt nor the **(D)** H95/S96 deer.

PrP^{CWD} deposition in kidney

Kidney histopathological samples were evaluated in 9 of the 10 CWD clinical deer. Positive immunolabeling was found in all evaluated kidney samples from wt/wt and S96/wt deer. PrP^{CWD} staining was consistently associated with arterial vessels, showing a periarterial and periarteriolar deposition. Most abundant immunolabeling was detected in the wall of the main renal artery and arcuate arteries, which locate at the junction of the renal cortex and the renal medulla and arise from interlobar arteries. This specific location of the immunolabeling could be due to the presence of nerve endings embedded in arterial walls. Interestingly, no PrP^{CWD} deposits were found in any of the evaluated kidney samples from the H95/wt and the H95/S96 deer (Table 2, Figure 6). In addition to the PrP^{CWD} aggregates observed in arterial vessels, one wt/wt deer showed also strong PrP^{CWD} deposition associated with foci of inflammatory cells (D1), which were compatible with a moderate nephritis (Figure 7).

PrP^{CWD} deposition in salivary glands

PrP^{CWD} deposits found in salivary glands were mild and scattered. Positive immunolabeling was found in the interstitial tissue between acini, whereas no intracellular deposits were detected in acinar cells or within salivary ducts in any of the samples evaluated. Parasympathetic ganglia neurons immersed in the salivary gland tissue from a wt/wt deer (D4), presented strong intraneuronal immunolabeling (Figure 6C). IHC deposits were observed in parotid and submandibular salivary glands, whereas all the evaluated sublingual glands samples were negative for PrP^{CWD} immunostaining. Deposition in the interstitial tissue was especially evident in the submandibular salivary gland of D7 (Figure 6D). Positive immunolabeling was detected in at least one salivary gland sample from all clinically affected deer with the exception of H95/wt and H95/S96 deer, which showed no PrP^{CWD} deposits in any of the salivary glands evaluated.

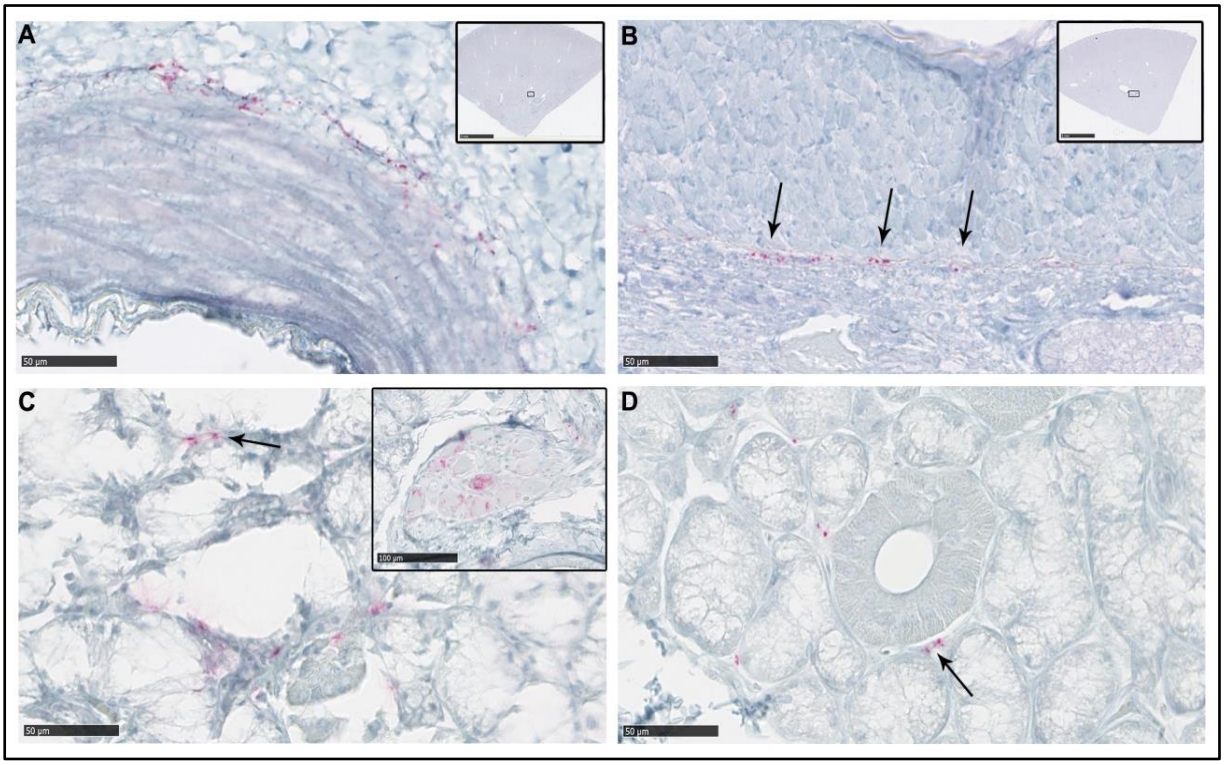

Figure 6: PrP^{CWD} deposition detected by IHC in the kidney and salivary glands of white-tailed deer of different *PRNP* genotypes infected with CWD. (A) Kidney from a wt/wt deer and a **(B)** S96/wt deer showing periarterial PrP^{CWD} deposition in arcuate arteries (arrows). Inserts show the specific location of these arteries in the histopathological sample. **(C)** Salivary gland from a wt/wt deer showing positive PrP^{CWD} immunolabeling in the interstitial tissue between acini (arrows). PrP^{CWD} immunolabeling was also detected in the ganglion neurons immersed in the salivary gland sample (insert picture). **(D)** Salivary gland from a S96/wt deer. Positive immunolabeling was detected in the same location as for wt/wt deer (arrow). No PrP^{CWD} deposition was observed in the kidneys or salivary glands of deer expressing the H95-PrP^{C}

PrP^{CWD} deposition in other organs

PrP^{CWD} immunolabeling was detected in the lungs of D1. This deer showed mild lung interstitial inflammation and edema. PrP^{CWD} aggregates were observed to be associated with inflammatory cell foci and in the cells of the bronchiole epithelium. This deer also showed, as mentioned previously, positive immunolabeling related to the accumulation of inflammatory cells in the kidney. Eye samples were collected from all deer included in this study. In addition to the positive immunolabeling of the optic nerve, described above, all deer were also positive for PrP^{CWD} IHC deposition in retina. All collected liver samples from the clinically affected deer were IHC negative (Table 2).

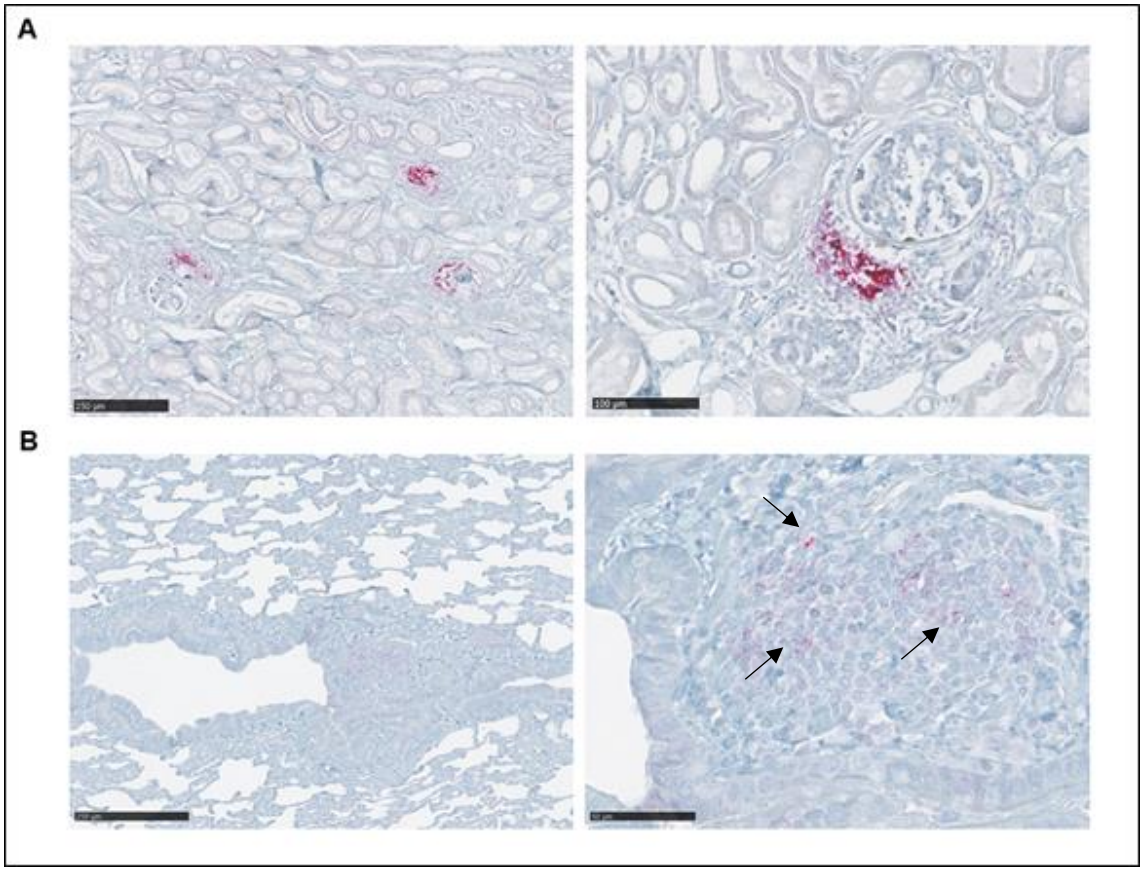

Figure 7: **PrP^{CWD} deposition detected by IHC in the kidney (A) and the lung (B) of a wt/wt deer which presented signs of inflammation (D1). (A)** A strong PrP^{CWD} deposition associated with foci of inflammatory cells was observed in this deer. Those foci of inflammation were generally found in the proximity of the glomeruli. **(B)** A moderate interstitial inflammation was also detected in this animal. PrP^{CWD} accumulation was observed associated with inflammatory cells (arrows).

Discussion

We found that the intensity and distribution of PrP^{CWD} deposits in brain and peripheral tissues of *PRNP* polymorphic (.i.e, expressing different PrP^{C} primary structures) white-tailed deer was distinct from Q95G95(wt) homozygous deer exposed to the same prion strain (i.e, Wisc-1). We have previously shown that H95 and S96 *PRNP* polymorphisms play a key role in CWD susceptibility, increasing survival periods and having dramatic effects on the propagation of CWD strains (Johnson *et al.*, 2003; O'Rourke *et al.*, 2004; Johnson *et al.*, 2006a; Johnson *et al.*, 2011; Duque Velasquez *et al.*, 2015; Herbst et al.;, 2017).

Our results show that deer expressing the H95-PrP^{C} presented a more limited peripheral distribution of PrP^{CWD} compared to wt/wt and S96/wt deer. Under identical experimental conditions and disease stage, the number of organs with positive immunolabeling was reduced in deer with H95-PrP^{C} allelotypes (Johnson *et al.*, 2011). The most significant differences in PrP^{CWD} deposition between deer with different *PRNP* genotypes were found in pancreas, heart, kidney and intestine samples. Both deer expressing the H95-PrP^{C} showed no immunolabeling or reduced accumulation of PrP^{CWD} aggregates in these tissues.

The presence of PrP^{CWD} in endocrine tissues has been previously described in the adrenal medulla, the pituitary gland and islets of Langerhans in the pancreas of CWD-affected cervids (Sigurdson *et al.*, 2001; Fox *et al.*, 2006), results that agree with our observations in wt/wt and S96/wt deer with CWD. Only deer of these genotypes (Figure 3A, B) showed moderate to strong chromagen deposition in islets of Langerhans, which are innervated by the vagus nerve (Loewy *et al.*, 1994; Sigurdson *et al.*, 2001). Differences in adrenal glands immunolabeling were minor between deer expressing PrP^{C} allotypes (i.e, host with two different PrP primary structures). However and consistent with wt/wt genotype being the cognate microenvironment for Wisc-1 prion replication, deer of this genotype presented more abundant and widespread PrP^{CWD} deposition, radiating from the adrenal medulla to the cortex.

The distribution of PrP^{CWD} aggregates was also limited in heart samples of animals expressing the H95-PrP^{C}. Heart samples were collected in D6 (S96/wt), D9 (H95/wt) and D10 (H95/S96). PrP^{CWD} accumulation was detected in multiple heart samples collected from D6, affecting separated groups of myocytes (Figure 4A). Conversely, no immunolabeling was observed in any heart sample from deer expressing the H95-PrPC (Figure 4B, C).

A distinct pattern of distribution of PrP^{CWD} was also observed for H95 carriers in intestinal tissues. It is not surprising that all evaluated deer presented strong immunolabeling in gut-associated lymphoid and nervous tissues, because it is known that, when the prion infection occurs via the oral route, these are some of the first sites where PrP^{d} accumulates (Sigurdson *et al.*, 1999; Andreoletti *et al.*, 2000; Fox *et al.*, 2006; Hoover *et al.*, 2017). Nevertheless, H95/wt and H95/S96 deer, which presented the longest incubation periods (Johnson *et al.*, 2011), showed more restricted or localized PrP^{CWD} accumulation (Figure 5). These findings however, cannot be associated with excretion of prions or absence of infectivity in these tissues. *In vitro* studies have shown that cervids shed prions early after oral CWD infection and polymorphisms linked to CWD delayed progression do not completely impede prion shedding in feces in preclinical stages of the disease (Cheng *et al.*, 2016; Plummer et al., 2018).

PrP^{CWD} deposits were observed in renal tissues of all wt/wt and S96/wt deer evaluated in the present study, whereas no immunopositive material was found in any of the kidney samples collected from deer expressing the H95 allele (Figure 6). Immunohistochemical detection of PrP^{CWD} in kidneys of CWD-infected white-tailed deer has only been reported in ectopic lymphoid follicles (Fox *et al.*, 2006; Hamir *et al.*, 2006). However, the presence of PrP^{CWD} in renal tissues has been demonstrated by sPMCA (Haley *et al.*, 2011) and it has been shown that CWD-infected cervids can shed infectious prions in urine (Haley *et al.*, 2009; John *et al.*, 2013; Henderson *et al.*, 2015), although the proximal source of PrP^{CWD} in urine is not known (Haley *et al.*, 2011). In the present study, PrP^{CWD} positive immunolabeling of kidney samples was detected along the wall of the renal arteries, especially in the main renal artery and the wall of arcuate arteries (Figure 6A, B). In scrapie-affected sheep, prion deposition has been found in renal papillae and renal corpuscles (Siso *et al.*, 2008; Garza *et al.*, 2014). Periarterial and periarteriolar immunolabeling could be due to a spread of prions through peripheral nerves (Siso *et al.*, 2008) since the wall of these renal arteries is strongly innervated and sympathetic nerve fibers from the renal plexus enter the kidney accompanying the branches of the main renal artery. To our knowledge, this is the first description of PrP^{CWD} deposition, detected by conventional techniques, in renal arteries of CWD-infected deer.

D1 also presented intense PrP^{CWD} immunolabeling in the renal cortex associated with accumulations of inflammatory cells (Figure 7). It has been demonstrated that inflammatory processes affect prion pathogenesis and peripheral accumulation (Heikenwalder *et al.*, 2005;

Ligios *et al.*, 2005), and that chronic nephritis triggers prionuria in prion infected mice (Seeger *et al.*, 2005). In addition, PrP^{CWD} shedding has been reported in CWD infected deer presenting with inflammatory kidney disease (Haley *et al.*, 2009). We cannot predict the effect of prion accumulation in the arterial walls on prionuria, however, we have observed that inflammatory kidney conditions greatly increase PrP^{CWD} deposition in renal tissues from deer with CWD, which might increase shedding of PrP^{CWD} within urine. This deer also showed PrP^{CWD} accumulation in the lungs associated with inflammatory cell foci and in the bronchiolar epithelium. PrP accumulation in the lung related to inflammatory conditions and in the epithelium of the bronchioles has been previously described in scrapie-affected sheep (Salazar *et al.*, 2010; Maestrale *et al.*, 2013; Garza *et al.*, 2014). Deer in our study were housed indoors with ample access to clean food and water (Johnson et al., 2011). It is likely that deer in the wild would be at greater risk of coincident infections and commensurate inflammation that may impact the effect of protective alleles on susceptibility to CWD, tissue colonization by CWD prions and shedding.

Regarding organs related to excreta production, certain differences in PrP^{CWD} deposition were found in salivary glands between deer genotypes. PrP^{d} in salivary glands, detected by conventional techniques, has been described in scrapie-affected sheep (Vascellari *et al.*, 2007) and in the serous epithelial cells of the submandibular salivary gland in experimentally infected red deer (Balachandran *et al.*, 2010). Likewise, considerable PrP^{CWD} amplifying activity, similar to that observed in brain, accumulates in salivary glands of cervids with CWD (Haley *et al.*, 2011). All positive salivary gland samples were collected from wt/wt or S96/wt deer. Although the deposition of PrP^{CWD} shown by these genotypes was scant and the number of evaluated samples was limited (Table 2), no positive salivary glands samples were observed in deer expressing H95-PrP^{C}. Intense PrP^{CWD} immunolabeling in ganglia cells immersed in the salivary gland tissue was observed in one animal (D4) (Figure 6C).

Our observations suggest that deer expressing H95-PrP^{C} have reduced centrifugal trafficking via descending nerves into tissues, regarding the specific location observed for PrP^{CWD} aggregates in several peripheral tissues (i.e. salivary glands, pancreas, heart and kidney). This is consistent with previous observations in different experimental prion infections (Kimberlin *et al.*, 1983; Sigurdson *et al.*, 2001; Jewell *et al.*, 2006; Crozet *et al.*, 2007; Siso *et al.*, 2008; Garza *et al.*, 2014). However, it has been suggested that, in initial

stages of CWD infection, PrP^{CWD} may be trafficked via blood (Hoover *et al.*, 2017), and PrP^{CWD} infectivity has been demonstrated in blood components (Mathiason *et al.*, 2006; Mathiason *et al.*, 2010). Therefore, we cannot exclude the hematogenous route as a complementary pathway of prion dissemination.

None of the clinically affected deer presented positive immunolabeling in any of the skeletal muscle samples evaluated, including the tongue. The absence of PrP^{d} accumulation in skeletal muscles detectable by IHC techniques has been reported in both naturally and experimentally prion infected deer (Spraker *et al.*, 2002; Hamir *et al.*, 2004). Nevertheless, although we did not detect PrP^{CWD} deposition in skeletal muscle samples in this experimental Wisc-1 transmission to white-tailed deer, we cannot assume the complete absence of prion accumulation. Others have demonstrated the presence of PrP^{CWD} in skeletal muscles by bioassay (Angers *et al.*, 2006), Western Blot, PMCA and tissue-blotting (Daus *et al.*, 2011).

The presence of prion deposits in skeletal muscles has been reported in neuromuscular spindles, which are strongly innervated structures (Andreoletti *et al.*, 2004; Garza *et al.*, 2014) and, in the case of CWD infected white-tailed deer, in nerve fascicles (Daus *et al.*, 2011). Although most of the vagus nerve samples evaluated in the present study showed positive immunolabeling, sciatic nerve and brachial plexus samples presented sparse or no PrP^{CWD} deposits (Table 1). Our results are similar to those described by Sigurdson *et al.* 2001 in mule deer naturally infected with CWD. We did not detect any PrP^{CWD} deposition in forelimb and hindlimb skeletal muscle samples, not even in the neuromuscular spindles. Due to the fact that brachial plexus and sciatic nerve innervate, respectively, the forelimb and hindlimb muscles, and taking into account that prions can spread to these muscles via these neural pathways (Sigurdson *et al.*, 2001), we suggest that the scant or absent PrP^{CWD} immunolabeling in brachial plexus and sciatic nerve samples from deer in our study may relate to the absence of deposits in these groups of muscles.

Cumulative evidence supports the limiting effect of H95 and S96 PrP^{C} polymorphisms on susceptibility of deer to CWD (Johnson *et al.*, 2003; O'Rourke *et al.*, 2004; Johnson *et al.*, 2006a) and the length of the incubation period (Johnson *et al.*, 2011). These PrP^{C} allelic variants can modulate CWD propagation and the efficiency of intraspecies CWD transmission (Angers *et al.*, 2014; Duque Velasquez *et al.*, 2015). The passage of CWD prions in white-tailed deer expressing the H95-PrP^{C} led to the emergence of the novel CWD strain $H95^{+}$(Duque Velasquez *et al.*, 2015). This strain presented distinct transmission

properties, being able to propagate in resistant transgenic mice expressing deer S96-PrP^{C} or in non-transgenic C57BL/6 mice (Duque Velasquez *et al.*, 2015; Herbst *et al.*, 2017). Here we show that Wisc-1 propagation in deer expressing the H95-PrP^{C} also presented limited peripheral PrP^{CWD} accumulation.

It is known that the *PRNP* genotype can strongly influence the tropism and distribution of PrP deposits, widely demonstrated in sheep scrapie. In the present study, the observed effects of the H95 allele in prion distribution resemble those described for sheep expressing the resistance-associated allele ARR at codons 136, 154 and 171 of the prion protein. ARR/VRQ sheep, despite developing disease and accumulating PrP^{Sc} in the brain, present a much more limited and infrequent PrP^{Sc} distribution in lymphoid tissues compared to those with other susceptible genotypes (van Keulen *et al.*, 1996; Andreoletti *et al.*, 2000; Houston *et al.*, 2002; Jeffrey *et al.*, 2002; Ersdal *et al.*, 2003). This effect is likely due to a modulation of the prion pathogenesis exerted by certain resistance-associated polymorphisms (Gonzalez *et al.*, 2010; Gonzalez *et al.*, 2014) and it is not necessarily associated with the prolonged incubation period. ARR/VRQ sheep, despite showing shorter survival times than ARQ/ARQ animals, present a significantly limited tissue dissemination of PrP^{Sc} (Gonzalez *et al.*, 2014).

The similarities in peripheral PrP^{CWD} accumulation between H95/wt and H95/S96 deer indicate a limiting role for the H95 amino acid substitution on the production and/or accumulation of PrP^{CWD}. Similar observations have been made in goats expressing methionine (M) at codon 142, which show reduced prevalence of scrapie infection and a lower tendency to accumulate PrP^{Sc} outside the brain compared to 142 isoleucine homozygotes (Gonzalez *et al.*, 2009; Balachandran *et al.*, 2010). By contrast, our findings suggest that H95-PrP^{C} does not affect lymphoreticular system involvement in CWD-affected deer (Table 1).

Influences of genotype on PrP^{CWD} deposition pattern have been also observed in experimentally infected mule deer, with 225SF deer presenting with milder PrP^{CWD} accumulation and more limited tissue distribution of PrP^{CWD} aggregates than 225SS animals at identical intervals post-inoculation. However, the 225SF amino acid variant was suggested to delay, rather than to limit, PrP^{CWD} tissue accumulation (Fox *et al.*, 2006). Similar observations have been made in white-tailed deer expressing the S96 allele, which show

significant lower PrP^{CWD} immunostaining in brain and lymphoid tissues, which was correlated to a slower disease progression (Johnson *et al.*, 2006a; Hoover *et al.*, 2017).

Considering that S96/wt and wt/wt deer presented very similar PrP^{CWD} deposition patterns, we emphasize the importance of the H95 polymorphism as a driver of the disease phenotype. The interference exerted by H95-PrP^{C} in the replication and spread of Wisc-1 prions may relate to the biology of resistance-associated PrP^{C} polymorphisms and the evolution of CWD prion strains (Duque Velasquez *et al.*, 2015; Herbst *et al.*, 2017).

Given the diversity of CWD agents is expanded in deer expressing PrP^{C} polymorphisms (Duque- Velasquez et al., 2015; Herbst et al., 2017, Hannaoui et al., 2017), our findings cannot be generalized to include all potentially existing CWD strains which will likely have distinct host specific interactions The disease characteristics, including the lesion distribution and the IHC phenotype of PrP^{d} accumulation, are strongly influenced by the infecting prion strain (Bruce *et al.*, 1991; Bessen and Marsh, 1994; Jeffrey *et al.*, 2003; Bartz *et al.*, 2005). Thus, it is possible that the milder PrP^{CWD} deposition, consistent with the reduced amounts of PrP^{CWD} by WB (Johnson et al., 2011), observed in certain brain areas of the H95/S96 deer (Figures 1 and 2) might be in part due to an intra-species transmission barrier phenomenon, which is strongly determined by the compatibility between the strain of the infecting prion and the PrP^{C} sequence of the host (Scott *et al.*, 1989; Telling *et al.*, 1996; Collinge, 2001). Deer in the present study were orally inoculated with Wisc-1 prions (Duque Velasquez *et al.*, 2015), a CWD source obtained from animals homozygous for the wt *PRNP* allele (Johnson *et al.*, 2011). Therefore, the transmission of the Wisc-1 strain into the H95/S96 deer involves an adaptation of the infectious agent, and it could partially explain the lower PrP^{CWD} accumulation shown by this animal in various brain areas, considering that the expression of wt-PrP^{C} favors the propagation of the Wisc-1 strain (Duque Velasquez *et al.*, 2015). In addition, it was found that the PrP^{CWD} accumulated in the H95/S96 animal [$H95^{+}$ CWD prions (Duque Velasquez *et al.*, 2015)] has lower resistance to PK than that from animals with at least one wt allele (Johnson *et al.*, 2011). Differences in strain resistance to proteolytic degradation are known to be in part responsible for certain variations observed between the results obtained in diagnostic tests. As an example, atypical/Nor98 scrapie isolates are highly sensitive to PK digestion when compared with classical scrapie strains, which leads to inconsistent results using confirmatory diagnostic methods, including IHC (Buschmann *et al.*, 2004). Therefore, this particular characteristic of $H95^{+}$ prions may also explain the lower immunolabeling observed in the brain of the H95/S96 deer since this

PrP^{CWD} could be partially destroyed during the IHC protocol used. Nevertheless, it has also been suggested that the disease phenotype, which includes the IHC profile of PrP^{d} accumulation in the brain, depends not on a single factor, but on complex interactions between the infecting agent or strain and host factors, including its genotype (Gonzalez *et al.*, 2012). We can correlate these findings with the differences observed between deer genotypes with respect to the PrP^{CWD} deposition in the cerebellum (Figure 2).

The present study indicates that the expression of CWD resistance-associated polymorphisms, as observed in other prion diseases, is able to limit the distribution of PrP^{CWD} aggregates in a wide variety of tissues and supports previous findings on the importance of the deer *PRNP* genotype on the modulation and adaptation of CWD strains.

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DISCUSIÓN GENERAL

Las enfermedades priónicas o EET pueden tener un origen esporádico, familiar o adquirido, sin embargo, todas ellas comparten una serie de características comunes. El hecho patogénico principal, común a todas las formas de las enfermedades priónicas es la conversión post-traduccional de la PrP^{C} en la isoforma patógena PrP^{Sc} que al acumularse en el SNC de los individuos afectados produce neurodegeración. Así, independientemente de cuál sea el origen de la proteína patógena en el SNC, el desarrollo de una enfermedad priónica lleva indefectiblemente a la muerte del individuo.

Existen todavía numerosas incógnitas sobre los mecanismos que regulan la transmisibilidad y la patogenia de estos procesos. Esto, y considerando que existen distintos tipos de EET y una gran variedad de cepas productoras de cada uno de ellos, dificulta aún más la disponibilidad de un tratamiento válido, aún inexistente. Se han realizado numerosos estudios encaminados a identificar un tratamiento para las EET, los cuales han explorado diversas aproximaciones terapéuticas. Ya que se ha demostrado ampliamente el efecto modulador que ejerce el genotipo del gen *PRNP* del hospedador sobre la barrera de transmisión frente a las EET, ciertos residuos aminoacídicos presentes de forma natural en algunas especies y relacionados con la resistencia a las enfermedades priónicas han sido estudiados como posibles candidatos para el desarrollo de terapias génicas. Algunos de estos residuos aminoacídicos, además de ejercer un efecto protector cuando están presentes en la PrP^{C}, son capaces de conferirle a ésta un efecto dominante negativo. Una PrP^{C} con una determinada sustitución aminoacídica ejerce un efecto protector dominante negativo cuando es capaz de interferir con la PrP^{C} de tipo salvaje o *wild-type* cuando ambas se coexpresan en el mismo hospedador, inhibiendo el malplegamiento de ésta. Existen varios residuos aminoacídicos presentes de forma natural en algunas especies que han demostrado ejercer una inhibición dominante negativa en la replicación de los priones. Entre ellos se encuentran el R171 de la oveja (Kaneko *et al.*, 1997; Zulianello *et al.*, 2000; Perrier *et al.*, 2002; Geoghegan *et al.*, 2009) y los polimorfismos E219K y G127V, relacionados con la resistencia a las EET en la especie humana (Shibuya *et al.*, 1998; Perrier *et al.*, 2002; Asante *et al.*, 2015).

Sin embargo, no solo es el genotipo del gen *PRNP* el que determina la susceptibilidad a las EET, sino que la cepa priónica transmitida es también un factor clave, y la mayor parte de las proteínas dominantes negativas descritas han demostrado un potente efecto protector, pero sólo frente a determinadas cepas (Houston *et al.*, 2003; Atarashi *et al.*, 2006; Striebel *et al.*, 2011). Por ello, en los dos primeros estudios de esta tesis nos

planteamos evaluar el efecto protector de un residuo aminoacídico de la PrP^C del perro, el D163, cuando este se introduce en una PrP^C exógena. Diversos estudios han llegado a la conclusión de que los cánidos son los mamíferos más resistentes a las EET (Khan *et al.*, 2010; Vidal *et al.*, 2013) y, posteriormente se observó que este residuo aminoacídico en concreto podría ser en gran parte responsable de su escasa susceptibilidad (Fernandez-Borges *et al.*, 2017). Como ya se ha demostrado los ratones que expresan la PrP^C murina con este único cambio de aminoácido (ratones TgN158D) eran resistentes a la inoculación con distintas cepas priónicas (Fernandez-Borges *et al.*, 2017). En el primer estudio de esta tesis se evalúa si esta proteína murina mutada podría además actuar como una proteína dominante negativa y prevenir la formación de PrP^{Sc} cuando se coexpresa con la PrP^C murina *wild-type*. Para ello se inocularon ratones Tga20xN158D, que coexpresan ambas proteínas, con varias cepas murinas de scrapie (22L, RML y ME7) y EEB (301C). Los periodos de supervivencia, las lesiones neuropatológicas y el patrón de glicosilación de estos animales se compararon con los obtenidos en ratones Tga20xKO, que expresan un nivel similar de PrP^C *wild-type,* para lo que inoculados con las mismas cepas priónicasy se observó que la PrP N158D prolongó significativamente el periodo de supervivencia de los ratones Tga20xN158D para todas las cepas inoculadas, demostrando el efecto dominante negativo de la proteína mutada. Cabe señalar que el nivel de expresión de la PrP^C *wild-type* en los ratones Tga20xN158D es aproximadamente el doble que el nivel de expresión de la proteína dominante negativa y, dado que se ha observado que las proteínas dominantes negativas suelen ejercer su efecto protector de una forma dosis-dependiente (Priola *et al.*, 1994; Perrier *et al.*, 2002; Geoghegan *et al.*, 2009), es probable que la prolongación del periodo de supervivencia hubiese sido mucho mayor si ambas proteínas se expresaran a un nivel equimolecular.

En el segundo estudio de esta tesis se evaluó el efecto de esta misma sustitución N>D en ratones TgVole que sobreexpresan la PrP^C I109 del *bank vole,* lo cual ocasiona que estos ratones transgénicos desarrollen una EET de forma espontánea, ya que la PrP^C de esta especie es muy propensa al malplegamiento (Watts *et al.*, 2012; Di Bari *et al.*, 2013). El objetivo principal de este estudio fue comprobar si este cambio aminoacídico, que produce total resistencia en el ratón (Fernandez-Borges *et al.*, 2017), era capaz de proteger frente a las enfermedades priónicas cuando se introduce en un modelo muy susceptible a las EET. Por ello se inocularon ratones TgVole-N159D, que sobreexpresan la PrP^C I109 del *bank vole* con la sustitución característica del perro, y los periodos de supervivencia y características

neuropatológicas de estos animales se compararon con los obtenidos en ratones TgVole sin la sustitución. Con el fin de corroborar la capacidad protectora del cambio aminoacídico frente a distintas cepas, estos ratones se inocularon con dos cepas de muy diferentes características: la cepa clásica CWD-vole, la cual es además la cepa priónica más rápida que se conoce con un periodo de supervivencia en el *bank vole* de 35 días (Di Bari *et al.*, 2013); y la cepa atípica Sp-TgVole, originada por el malplegamiento espontáneo de la PrP^{C} I109 del modelo TgVole. Como resultado se observó que si bien los animales TgVole-N159D sí desarrollaron la enfermedad, lo hicieron mostrando un periodo de supervivencia significativamente más largo que los controles TgVole, siendo este retraso en la aparición de signos clínicos especialmente notable con la cepa CWD-vole (108% de retraso). Asimismo, el cambio N159D fue capaz de retrasar la aparición de la enfermedad espontánea en ratones TgVole-N159D sin inocular. Los ratones inoculados con la cepa atípica Sp-TgVole desarrollaron características neuropatológicas casi idénticas a las observadas en ratones TgVole-N159D y TgVole sin inocular, si bien sucumbieron a la enfermedad en un periodo de tiempo más corto. Estos resultados parecen sugerir que la inoculación del aislado Sp-TgVole acelera la aparición de la enfermedad espontánea, debido a un fenómeno de aceleración por inoculación. Este fenómeno se produce cuando un modelo transgénico desarrolla una enfermedad de forma espontánea, pero el proceso patológico puede ser acelerado por la inoculación exógena del agente (Fernandez-Borges *et al.*, 2013).

No sabemos con certeza cuáles son los mecanismos moleculares exactos por los que la sustitución N>D retrasa la propagación de los priones. Se ha sugerido que cuando ciertas PrPs portadoras de sustituciones protectoras se coexpresan junto con la PrP^{C} *wild-type*, estas moléculas de PrP mutadas pueden interferir con las interacciones entre los monómeros de PrP^{C} y PrP^{Sc} (Hope *et al.*, 1986; Prusiner *et al.*, 1990; Priola *et al.*, 1994) dando lugar a polímeros inestables de PrP^{Sc} (Priola *et al.*, 1994; Geoghegan *et al.*, 2009). Se ha propuesto por tanto que la introducción de estas sustituciones en la PrP^{C} dificulta el proceso por el cual la PrP^{C} se convierte en PrP^{Sc} (Priola *et al.*, 1994), ya que las diferencias entre la proteína mutada y la *wild-type*, aunque se trate de un aminoácido, pueden hacer que ambas proteínas sean estructuralmente incompatibles (Jahandideh *et al.*, 2015). Estudios anteriores han demostrado que el cambio N>D en la PrP produce alteraciones significativas en la superficie de la proteína (Fernandez-Borges *et al.*, 2017). Por ello, esta sustitución puede que produzca sus efectos protectores causando alteraciones en la PrP que retrasen la tasa de formación y la estabilidad de las nuevas moléculas de PrP^{Sc}, aunque serían necesarios otros estudios para

comprobar esta hipótesis. No obstante, podemos sugerir que la prolongación en el periodo de supervivencia observada en los ratones Tga20xN158D y TgVole-N159D parece no deberse a alteraciones de las propiedades de las cepas inoculadas causadas por la introducción de la sustitución aminoacídica en la PrP. Los ratones Tga20xN158D y TgVole-N159D mostraron características neuropatológicas muy similares a las desarrolladas por sus respectivos controles sin la sustitución. Por tanto, hemos observado que la introducción de este residuo aminoacídico en la PrP, característico de especies muy poco susceptibles a las EET, ejerce un efecto protector frente a cepas priónicas muy diversas, por lo que podría ser un buen candidato en el desarrollo de estrategias genéticas frente a las EET.

El concepto de especies resistentes a las EET surgió a partir de la crisis de la EEB ya que, a pesar de que el número de especies afectadas por enfermedades priónicas aumentó exponencialmente, no se detectaron casos de EET en ciertos grupos de mamíferos que también habían estado expuestos a productos contaminados con priones infectivos para los bovinos (Kirkwood *et al.*, 1993; Kirkwood and Cunningham, 1994; Sigurdson and Miller, 2003; Fernandez-Borges *et al.*, 2012). No obstante, la aparición de la vECJ en la especie humana fue el hecho que demostró la transmisibilidad entre especies distintas de las enfermedades priónicas era posible, lo que se convirtió en un motivo de preocupación para la salud pública. Además, se han detectado casos naturales de EEB en pequeños rumiantes (Eloit *et al.*, 2005; Spiropoulos *et al.*, 2011) y se ha comprobado que la patogenicidad de los priones de EEB para el ser humano aumenta al pasar a través de estas especies (Padilla *et al.*, 2011). Por ello, se ha considerado de gran importancia conocer los mecanismos implicados en la transmisión de las EET a la especie humana. El papel de la glicosilación de la PrP^{C} en la transmisión de las EET ha sido estudiado en profundidad. Los glicanos unidos a la proteína prion son indispensables para su tráfico intracelular y su localización en la membrana plasmática, mecanismo fundamental para que la infección priónica se produzca (Rogers *et al.*, 1990; DeArmond *et al.*, 1997; Lehmann and Harris, 1997; Korth *et al.*, 2000; Salamat *et al.*, 2011). Ya que la glicosilación de la PrP^{C} modula significativamente las interacciones entre ésta y la PrP^{Sc}, se ha sugerido que los glicanos podrían ser determinantes tanto en la conversión de la PrP^{C} en la forma patógena, como en la eficiencia de transmisión de los priones entre las especies (Priola and Lawson, 2001). Por ello en el tercer estudio de esta tesis evaluamos el efecto de la ausencia de glicanos en la PrP^{C} humana sobre la barrera de transmisión y el mantenimiento de las características de un amplio rango de cepas priónicas: SSBP/1 (scrapie clásico), scrapie atípico, ECC, BSE-L y BSE-H (EEB atípicas),

BSE-C (EEB clásica), EEB ovina, EEB porcina y vECJ. Se analizó la capacidad de propagación de estos aislados en un modelo humano que expresa una PrP^C humana sin glicosilar , la línea transgénica TgNN6h (Haldiman *et al.*, 2013). Para ello se utilizaron técnicas *in vitro* (PMCA) y se realizó un bioensayo en ratones TgNN6h y en ratones Tg340, los cuales expresan la PrP^C humana normalmente glicosilada (Padilla *et al.*, 2011). Tras 15 rondas consecutivas de PMCA, solo los priones relacionados con la EEB clásica, es decir, BSE-C, EEB ovina, EEB porcina y vECJ, se propagaron en el sustrato humano no glicosilado. Además, lo hicieron mostrando distintas eficiencias de propagación, similares a las descritas en transmisiones experimentales de las mismas cepas en modelos humanos que expresan una PrP^C normalmente glicosilada (Padilla *et al.*, 2011; Plinston *et al.*, 2011; Barria *et al.*, 2014). Asimismo, el hecho de que el resto de las cepas no pudieran ser propagadas en el sustrato TgNN6h sugiere que la barrera de transmisión humana frente a las EET se mantiene a pesar de la ausencia de glicanos de la PrP^C. Estos aislados de EEB se adaptaron *in vitro* al sustrato TgNN6h, y se inocularon *in vivo* en ratones TgNN6h, lo cual facilitó enormemente su transmisión a este modelo, ya que los animales murieron consecuencia de la enfermedad en aproximadamente 200 días, mientras que la inoculación de los aislados directos, a excepción del aislado de vECJ, no causó enfermedad en estos animales en un primer pase. Ello indica que la adaptación y estabilización de los priones por PMCA facilita el salto de la barrera de especie (Castilla *et al.*, 2008). Los ratones TgNN6h, a pesar de que expresan una PrP^C sin glicosilar, desarrollaron las características neuropatológicas y bioquímicas típicas de la EEB cuando se transmite al hombre o a modelos humanizados (Will *et al.*, 1996; Biacabe *et al.*, 2007; Beringue *et al.*, 2008; Padilla *et al.*, 2011), lo cual parece indicar que los glicanos unidos a la PrP^C no son indispensables para el mantenimiento de las características patobiológicas de la EEB, a diferencia de lo sugerido para otras cepas (Cancellotti *et al.*, 2013). Como se ha mencionado, observamos que la vECJ fue la única cepa que se transmitió en un primer pase a los ratones TgNN6h tras la inoculación directa del aislado. Estos resultados están de acuerdo con lo descrito en otros estudios en los que se indica que la transmisión de la vECJ a modelos humanizados es más eficiente que la de la EEB, a pesar de que la vECJ deriva de la EEB, lo que demuestra que es una cepa de propiedades muy estables tras la transmisión (Lasmezas *et al.*, 2001; Bishop *et al.*, 2006). Los ratones afectados por vECJ desarrollaron características neuropatológicas típicas de esta cepa, pero también acumularon grandes depósitos de amiloide en el encéfalo, lo que sugiere que al ser un primer pase, la cepa no está completamente adaptada al modelo y por tanto la neuropatología observada en los animales es más heterogénea. El bioensayo en ratones

TgNN6h y Tg340 sigue en curso, y los resultados que se obtengan en el mismo ayudarán a esclarecer este punto.

En la segunda parte de esta tesis se han incluido dos estudios diseñados para ampliar el conocimiento sobre la patogenia de las EET. En el primero de ellos (estudio nº 4) se evalúa el papel del estrés del retículo endoplásmico (RE) y de la degradación proteica por parte del sistema ubiquitino-proteasómico (UPS) en modelos murinos que desarrollan una EET de forma espontánea, mediante el uso de técnicas inmunohistoquímicas. Para ello se utilizaron muestras de encéfalo de ratones $TgU1^{+}/TgVole^{+}$ de distintas edades. Estos ratones, además de sobreexpresar la PrP^{C} I109 del *bank vole*, lo cual les provoca una EET espontánea, como se ha descrito anteriormente, también expresan el marcador Ub^{G76V}-GFP. Este marcador está constituido por una ubiquitina marcada con la proteína verde fluorescente GFP, lo cual permite monitorizar la degradación proteasómica *in vivo*, ya que el fallo del UPS provoca la acumulación de este marcador en estos ratones (Lindsten *et al.*, 2003). La proteína PDI se seleccionó como marcador de estrés del RE. La sobreexpresión de las proteínas de la familia de la PDI se ha descrito en las enfermedades priónicas (Yoo *et al.*, 2002; Hetz *et al.*, 2003; Torres *et al.*, 2015) sugiriendo que podría tratarse de un mecanismo que el organismo utiliza para intentar defenderse de la acumulación de proteínas malplegadas (Yoo *et al.*, 2002; Hetz *et al.*, 2003; Hetz *et al.*, 2005). Se sabe que el estrés crónico del RE produce una acumulación de PrP malplegada en el citosol (Kang *et al.*, 2006; Orsi *et al.*, 2006), que puede acumularse e inhibir la actividad del UPS (Kristiansen *et al.*, 2007). Se ha descrito que que en el curso de la patogenia de las EET adquiridas se produce una pérdida de la función del UPS (McKinnon *et al.*, 2016). En cambio, los estudios que se han realizado al respecto utilizando modelos de EET familiares han llegado a conclusiones muy dispares (Quaglio *et al.*, 2011; Wang *et al.*, 2011; Wang *et al.*, 2012). Por ello decidimos analizar estos procesos en las EET espontáneas mediante el estudio de la acumulación de PDI y Ub^{G76V}-GFP en ratones $TgU1^{+}/TgVole^{+}$ jóvenes (preclínicos) y adultos (clínicos) y comparamos los resultados con los obtenidos en ratones $TgU1^{+}/TgVole^{-}$ de edades similares, los cuales, al no expresar la PrP^{C} del *bank vole*, no desarrollan una EET espontánea y por tanto actúan como controles del estudio. Si bien los ratones $TgU1^{+}/TgVole^{+}$ que presentaron signos clínicos mostraron mayor acumulación de ambos marcadores con respecto a los demás grupos de ratones en todas las áreas encefálicas estudiadas, sólo se observaron diferencias significativas en determinadas áreas. Se detectaron depósitos de PDI y Ub^{G76V}-GFP en todos los animales sanos, tanto jóvenes como adultos, si bien los animales adultos presentaron, en general, una

mayor acumulación de estos marcadores, lo cual podría indicar que se produce cierto grado de estrés del RE y de disfunción del UPS como consecuencia del envejecimiento. No se observaron diferencias significativas entre los animales preclínicos y sus controles respecto a la acumulación de PDI y Ub^{G76V}-GFP, lo que sugiere que el estrés del RE y la pérdida de la función del UPS no son acontecimiento que se producen tempranamente en la patogenia de las EET esporádicas, contrariamente a lo descrito en otras formas de EET (Hetz *et al.*, 2005; McKinnon *et al.*, 2016). Sin embargo, observamos que los depósitos de Ub^{G76V}-GFP en los ratones $TgU1^{+}$/$TgVole^{+}$ clínicos y preclínicos presentaban una morfología distinta a la de los depósitos de Ub^{G76V}-GFP detectados en los animales sanos. Estas diferencias se debían en gran medida a que una gran parte de las células en las que se observaron estos depósitos en los ratones $TgU1^{+}$/$TgVole^{+}$ eran astrocitos reactivos. La intensa acumulación de Ub^{G76V}-GFP en astrocitos reactivos se ha descrito anteriormente (McKinnon *et al.*, 2016) y se ha demostrado que la acumulación de GFAP produce una disminución de la actividad del proteasoma (Tang *et al.*, 2006), por lo que este patrón de acumulación podría ser un fenómeno ligado a la astrogliosis que se desarrolla en el curso de las enfermedades priónicas. A pesar de que es necesario complementar nuestros resultados con otros estudios bioquímicos y moleculares, podemos concluir que, al igual que se ha descrito en otros estudios (Yoo *et al.*, 2002; Quaglio *et al.*, 2011), tanto el estrés del RE como la disminución de la degradación proteica por parte del UPS parecen ser hechos secundarios asociados con el desarrollo de los cambios neuropatológicos en las EET esporádicas, en vez de mecanismos clave en la patogenia de estos procesos.

Finalmente, en el quinto estudio de esta tesis, evaluamos el efecto que ejercen ciertos polimorfismos del gen *Prnp* asociados con la resistencia a la ECC en el depósito de la proteína prión patológica en diez ciervos de cola blanca (*Odocoileus virginianus*) inoculados por vía oral con este agente. La ECC es una enfermedad priónica que afecta a diversas especies de cérvidos de Norteamérica y Corea del Sur, y, en el año 2016, se ha descrito por primera vez en Europa (Benestad *et al.*, 2016). Actualmente, se han encontrado varios casos de la enfermedad en Noruega y Finlandia, siendo considerada una enfermedad emergente en el continente europeo. La ECC es altamente contagiosa, unido a que es la única EET que afecta a animales de vida silvestre y a la gran capacidad de persistencia del agente en el ambiente, dificulta enormemente el control y la erradicación de esta enfermedad (Miller *et al.*, 2004; Johnson *et al.*, 2006; Schramm *et al.*, 2006; Almberg *et al.*, 2011). Se ha demostrado ampliamente, especialmente en el caso del scrapie, que ciertos polimorfismos

asociados con la resistencia a las EET son capaces de modular la patogenia de la enfermedad y afectar a la distribución de los priones en el organismo del hospedador (van Keulen *et al.*, 1996; Andreoletti *et al.*, 2000; Houston *et al.*, 2002; Jeffrey *et al.*, 2002; Ersdal *et al.*, 2003; Gonzalez *et al.*, 2010; Gonzalez *et al.*, 2014b). En este estudio analizamos el patrón de distribución inmunohistoquímico de la PrP^{CWD} en el encéfalo y en los tejidos periféricos de ciervos de distinto genotipo para el gen *Prnp*: Q95G96/Q95G96 (wt/wt), S96/wt, H95/wt y H95/S96, todos ellos en la fase terminal de la enfermedad tras la inoculación oral de la cepa de ECC Wisc-1 (Johnson *et al.*, 2011; Duque Velasquez *et al.*, 2015). Se observó que los animales que expresaban el alelo H95 presentaban una distribución periférica de PrP^{CWD} mucho más limitada que los animales wt/wt y S96/wt. Las diferencias más significativas se observaron en el páncreas, corazón, riñón e intestino, pues los animales H95, o bien no mostraban ningún tipo de depósito de PrP^{CWD} o bien acumularon una cantidad mucho menor de PrP^{CWD} en estos tejidos en comparación con el resto de los genotipos. Además, en este estudio se describe por primera vez el depósito de PrP^{CWD} en arterias renales detectado por técnicas de diagnóstico convencionales. Se sugiere, por tanto, que en esta transmisión experimental de la cepa Wisc-1, la expresión de la PrP^{C} H95 ha limitado la distribución periférica de la PrP^{CWD} en el estadio terminal de la ECC, un efecto similar al observado para otros polimorfismos de resistencia en otras enfermedades priónicas (Gonzalez *et al.*, 2009; Balachandran *et al.*, 2010; Gonzalez *et al.*, 2014a). Estos resultados refuerzan la importancia del polimorfismo H95 en la resistencia a la ECC (Johnson *et al.*, 2011) y en la modulación y adaptación de nuevas cepas de ECC capaces de transmitirse a hospedadores resistentes (Duque Velasquez *et al.*, 2015; Herbst *et al.*, 2017). Asimismo, a pesar de que el patrón de depósito de PrP^{CWD} en el encéfalo de todos los animales fue similar, se observaron diferencias en ciertas áreas. Por un lado, se detectó que el animal H95/S96, portador de los dos polimorfismos asociados a resistencia, acumuló una cantidad significativamente menor de PrP^{CWD} en las regiones más rostrales del encéfalo. Esto podría deberse a un fenómeno de barrera de transmisión intraespecie, ya que los priones Wisc-1 proceden de animales wt/wt (Duque Velasquez *et al.*, 2015), o bien a que los priones generados en este animal presentan una menor resistencia a la PK (Johnson *et al.*, 2011). Por otro lado, se observó que cada genotipo tenía un perfil de depósito distinto en el cerebelo. En otros estudios se ha sugerido que el fenotipo neuropatológico de las enfermedades priónicas no sólo depende de un factor, como el genotipo del gen *PNRP* o la cepa, sino que está determinado por complejas interacciones entre el agente infeccioso y otros factores propios del hospedador (Gonzalez *et al.*, 2012), lo que podría explicar lo observado en el cerebelo de estos animales.

Los resultados obtenidos en el conjunto de esta tesis corroboran la importancia del estudio de los factores implicados en la transmisión y la patogenia de las EET, destacando el papel fundamental que juega la secuencia de aminoácidos del gen *PRNP* en la modulación de la susceptibilidad a estos procesos. Existen numerosos factores no conocidos de estas enfermedades, por lo que el estudio de variantes aminoacídicas relacionadas con la resistencia y su posible aplicabilidad terapéutica resultan de gran interés. No hay que olvidar que el periodo de incubación de las enfermedades priónicas en el ser humano puede ser de décadas, por lo que cualquier factor capaz de prolongar el periodo de supervivencia en modelos experimentales podría ser de gran utilidad si extrapolamos los resultados a la especie humana.

Asimismo, es indispensable profundizar en el estudio de la patogenia de estos procesos. Sólo conociendo los mecanismos patogénicos que determinan la aparición de las EET es posible intentar desarrollar un tratamiento curativo o preventivo para estas enfermedades, elaborando estrategias destinadas a actuar sobre distintas rutas moleculares.

CONCLUSIONES/CONCLUSIONS

1. La sustitución aminoacídica N158D en la PrP murina, un cambio aminoacídico característico de los cánidos, confiere un efecto dominante negativo a la PrP mutada, capaz de atenuar la conversión de la PrP^{C} en la PrP^{Sc}.
2. La sustitución aminoacídica N>D es capaz de conferir un efecto protector frente a la propagación de cepas priónicas de muy distintas características, tanto en modelos experimentales muy susceptibles a las enfermedades priónicas como en modelos transgénicos que desarrollan EET espontáneas.
3. La expresión del cambio aminoacídico N>D parece no afectar a las características patobiológicas de las cepas priónicas inoculadas, sugiriendo que el incremento en el periodo de supervivencia observado en los ratones que lo expresan no se debe a modificaciones de cepa causadas por el cambio aminoacídico de la proteína mutada. Por ello, este cambio aminoacídico podría ser un candidato prometedor en el desarrollo de estrategias para futuras terapias génicas frente a las enfermedades priónicas.
4. La inoculación de la cepa Sp-TgVole, originada por el malplegamiento espontáneo de la PrP^{C} I109 del *bank vole*, acelera la aparición de la enfermedad espontánea, reproduciendo sus características neuropatológicas, indicando que este proceso patológico puede ser acelerado por la inoculación exógena del agente.
5. Sólo los priones relacionados con la EEB son capaces de propagarse cuando se utiliza como sustrato una PrP^{C} humana sin glicosilar, y además lo hacen mostrando distintas eficiencias de propagación, lo que sugiere que la barrera de transmisión de los priones al hombre no se ve alterada por la ausencia de glicanos en la proteína prion humana.
6. La compatibilidad entre las secuencias de aminoácidos de inóculo y sustrato y otras ligeras adaptaciones de la vECJ al ser humano podrían explicar la mayor eficiencia de propagación de este aislado en una PrP^{C} humana sin glicosilar con respecto a los demás aislados de EEB.
7. La adaptación de los aislados de EEB por PMCA al sustrato humano no glicosilado facilita enormemente la transmisión de los mismos a ratones que expresan este tipo de PrP^{C}, mientras que la inoculación directa de los aislados requiere de periodos de incubación más largos.
8. Las características neuropatológicas de la EEB se mantienen tras ser transmitidas a un modelo transgénico humano que expresa una PrP^{C} sin glicosilar, indicando

que la ausencia de glicanos en la PrP^{C} humana no altera las características de la EEB al ser transmitida.

9. En ratones que desarrollan una EET espontánea, el estrés del retículo endoplásmico y la pérdida de la función proteolítica del sistema ubiquitino-proteasómico se detectan sólo en ciertas áreas encefálicas, por lo que parecen hechos colaterales asociados con la neurodegeneración, más que mecanismos esenciales en la patogenia de las EET espontáneas.
10. La expresión de histidina en el codón 95 (H95) de ciervos de cola blanca, infectados oralmente con la cepa de ECC Wisc-1, es capaz de limitar la distribución de PrP^{CWD} a los tejidos periféricos del mismo modo que se ha descrito en otras EET.

1. N158D substitution in murine PrP, an amino acid change characteristic of canids, confers a dominant negative effect on the mutated PrP, attenuating the conversion of PrP^{C} to PrP^{Sc}.
2. The amino acid substitution N> D is able to confer a protective effect against the propagation of prion strains of very different characteristics, both in experimental models highly susceptible to prion diseases and in transgenic models that develop a TSE spontaneously.
3. The expression of the amino acid change N> D does not seem to affect the pathobiological characteristics of the inoculated prion strains, suggesting that the increase in the survival times observed in mice expressing this specific substitution is not due to strain modifications caused by the amino acid change of the mutated protein. Therefore, this amino acid change could be a promising candidate in the development of strategies for future gene therapies against prion diseases.
4. The inoculation of the Sp-TgVole strain, originated by the spontaneous misfolding of I109 bank vole PrP^{C}, accelerates the onset of the spontaneous disease, reproducing its neuropathological characteristics, indicating that this pathological process can be accelerated by the exogenous inoculation of the agent.
5. Only BSE-related prions are capable of propagating in a non-glycosylated human PrP^{C} substrate, showing different propagation efficiencies, suggesting that the human transmission barrier for prions is not altered in the absence of glycans in the human prion protein.
6. The compatibility between the amino acid sequences of inoculum and substrate and other slight adaptations of vCJD to humans could explain the greater propagation efficiency of this isolate in non-glycosylated human PrP^{C} compared to the other BSE-related isolates.
7. Adaptation of BSE isolates by PMCA to the non-glycosylated human substrate greatly facilitates transmission to mice expressing this form of PrP^{C}, whereas direct inoculation of the isolates requires longer incubation periods.
8. The neuropathological characteristics of BSE are maintained after transmission to a transgenic human model expressing a non-glycosylated PrP^{C}, indicating that the absence of glycans in human PrP^{C} does not alter the characteristics of BSE when it is transmitted.

9. In mice that develop a spontaneous TSE, endoplasmic reticulum stress and the impairment of the proteolytic function of the ubiquitin-proteasomal system are detected only in certain brain areas, therefore, both mechanisms seem to be collateral events associated with neurodegeneration, rather than essential mechanisms in the pathogenesis of spontaneous TSE.
10. The expression of histidine at codon 95 (H95) of white-tailed deer orally infected with the CWD strain Wisc-1 limits the distribution of PrP^{CWD} to peripheral tissues in a similar way to that described for other TSE.

RESUMEN/ABSTRACT

Las enfermedades priónicas han sido descritas en numerosas especies de mamíferos, pudiendo tener un origen espontáneo, familiar o adquirido. Sin embargo, ciertas especies, entre ellas los miembros de la familia *Canidae*, han demostrado presentar una escasa susceptibilidad a estos procesos. Algunas variantes aminoacídicas de la PrP^{C} son factores determinantes en la susceptibilidad a las enfermedades priónicas. Estudios anteriores utilizando modelos murinos transgénicos revelaron la importancia del cambio aminoacídico N>D, característico de los cánidos, en la resistencia a diversas cepas priónicas. El **estudio número 1** se diseñó con el objetivo de comprobar si la PrP con esta sustitución (PrP N158D) puede actuar como una proteína dominante negativa y prevenir la formación de PrP^{Sc} cuando se coexpresa con la proteína murina de tipo salvaje o *wild-type*. Los ratones transgénicos que coexpresaban la PrP mutada mostraron un incremento significativo del periodo de supervivencia (entre un 45 y un 113% de retraso en la aparición de la enfermedad), con respecto a ratones control que expresaban un nivel similar de PrP^{C} *wild-type*, tras la inoculación de distintas cepas priónicas murinas (22L, ME7, RML y 301C). Asimismo, los ratones que expresaban la PrP mutada no mostraron alteraciones en las características neuropatológicas y bioquímicas desarrolladas, indicando que la prolongación del periodo de supervivencia no se debió a cambios en las cepas inoculadas causadas por el cambio aminoacídico introducido. Estos resultados demuestran que esta sustitución específica de los cánidos proporciona a la PrP la capacidad de actuar como proteína dominante negativa, atenuando la conversión de la PrP^{C} en la forma patógena y retrasando la aparición de la enfermedad tras la inoculación de cepas priónicas de distintas características.

El **estudio número 2** surge como una continuación del primero. En este estudio se analiza el efecto de esta misma sustitución cuando se introduce en la PrP^{C} de una especie muy susceptible a numerosas enfermedades priónicas. Como modelos de alta susceptibilidad a las EET se seleccionaron ratones transgénicos que sobreexpresaban la PrP^{C} I109 del *bank vole*, la cual es tan susceptible al malplegamiento que su sola sobreexpresión ocasiona que estos ratones desarrollen una EET de forma espontánea, Así pues, en el segundo estudio se inocularon ratones TgVole-N159D, que sobreexpresan la PrP^{C} I109 del *bank vole* y son portadores de la sustitución aminoacídica de interés, con dos cepas priónicas distintas la CWD-vole y una cepa atípica de origen espontáneo. Como resultado de ello se observó que estos animales presentaron periodos de supervivencia de un 52 a un 108% más largos que los obtenidos en ratones TgVole sin la sustitución.

Además, este residuo aminoacídico fue capaz de prolongar el periodo de supervivencia, no sólo en los ratones inoculados, sino en en los que habían desarrollado la EET espontánea. Al igual que se observó en el estudio 1, no se detectaron diferencias entre las características neuropatológicas desarrolladas por ambos modelos transgénicos. Estos resultados demuestran que el cambio aminoacídico N>D característico de los cánidos produce un efecto protector frente a la propagación de priones de muy distinto origen, incluso cuando se expresa en una PrP^C muy susceptible al malplegamiento. Estos dos primeros estudios indican que la sustitución aminoacídica N>D, típica de especies poco susceptibles a las EET, podría ser una candidata interesante en el desarrollo de futuras terapias génicas frente a estas enfermedades.

Tras la crisis de la EEB y la aparición de la vECJ en la especie humana, el conocimiento de los mecanismos relacionados con la transmisibilidad de las EET entre especies se convirtió en un motivo de preocupación pública. La implicación de la glicosilación de la PrP^C en la conversión en forma patógena de la proteína y en la transmisión interespecie de los priones se ha estudiado ampliamente, si bien se han obtenido resultados controvertidos. En el **estudio número 3** se evalúa el efecto de la glicosilación de la PrP^C humana en la barrera de transmisión y en las propiedades de distintas cepas priónicas. Se utilizaron aislados de scrapie clásico, scrapie atípico, ECC, BSE-L y BSE-H (EEB atípicas), BSE-C (EEB clásica), EEB ovina, EEB porcina y vECJ en experimentos *in vitro* e *in vivo* con el fin de analizar la capacidad de propagación de estos aislados en un modelo que expresa una PrP^C humana sin glicosilar (línea transgénica TgNN6h). Sólo las cepas relacionadas con la BSE (BSE-C, EEB ovina, EEB porcina y vECJ) se propagaron en el sustrato humano no glicosilado, lo cual sugiere que la barrera de transmisión humana para las EET se mantuvo. En el bioensayo, a excepción de la vECJ, la inoculación directa de estos mismos aislados no provocó enfermedad en los ratones TgNN6h en un primer pase. Sin embargo, tras la adaptación al sustrato TgNN6h de tres aislados de EEB (bovina, ovina y porcina) por PMCA y la inoculación de estos en ratones se consiguió una transmisión muy eficiente. Los animales TgNN6h inoculados con los inóculos adaptados por PMCA o el aislado directo de vECJ desarrollaron las características neuropatológicas y bioquímicas clásicas de la EEB, lo cual sugiere que la ausencia de glicanos en la PrP^C humana no altera las características patobiológicas de la EEB.

Los mecanismos reguladores de la patogenia de las EET, especialmente los de las formas esporádicas de estos procesos, siguen siendo en su mayor parte desconocidos. Existen numerosos estudios que indican que la acumulación de proteínas malplegadas, no sólo en las enfermedades priónicas, sino en otras enfermedades neurodegenerativas, podrían inducir estrés del retículo endoplásmico y/o un bloqueo de la degradación proteica por parte del sistema ubiquitino-proteasómico. Por ello, en el **estudio número 4** analizamos por medio de técnicas inmunohistoquímicas dos marcadores relacionados con estos procesos, en muestras de encéfalo de modelos murinos de EET espontánea (TgVole). Asimismo, estos ratones expresaban el marcador Ub^{G76V}-GFP (línea TgU1), cuya acumulación indica una disfunción de la degradación proteasómica. Por tanto, la acumulación de Ub^{G76V}-GFP se seleccionó como marcador del bloqueo de la degradación del sistema ubiquitino-proteasómico y la proteína PDI como marcador de estrés del retículo endoplásmico. Se seleccionaron ratones $TgU1^{+}/TgVole^{+}$ de distintas edades para evaluar estos procesos en las fases preclínica y clínica de la enfermedad espontánea. Como controles se seleccionaron ratones $TgU1^{+}/TgVole^{-}$ de similares edades. Los animales $TgU1^{+}/TgVole^{+}$ clínicos mostraron una acumulación de PDI y Ub^{G76V}-GFP significativamente mayor que los controles de la misma edad en determinadas áreas del encéfalo. Sin embargo, en los ratones preclínicos $TgU1^{+}/TgVole^{+}$ no se detectó incremento significativo del estrés del retículo endoplásmico ni pérdida significativa de la función del proteasoma en ninguna de las regiones del encéfalo analizadas. Por ello, ninguno de estos procesos parece ocurrir tempranamente en la patogenia de las formas espontáneas de las EET. Se observó, asimismo, que los ratones $TgU1^{+}/TgVole^{+}$, especialmente los clínicos, mostraron una intensa acumulación de Ub^{G76V}-GFP en astrocitos reactivos. Estos resultados preliminares parecen indicar que tanto el estrés del retículo endoplásmico como la disminución de la degradación proteica por parte del proteasoma son mecanismos secundarios que aparecen asociados con el desarrollo de los cambios neuropatológicos en el encéfalo en las EET espontáneas, en lugar de mecanismos clave en la patogenia de estas enfermedades.

Finalmente, en el **estudio número 5** analizamos el patrón de distribución inmunohistoquímico de la PrP^{CWD} en el SNC y en un amplio rango de tejidos periféricos procedentes de diez ciervos de cola blanca (*Odocoileus virginianus*) de distinto genotipo para el gen *PRNP*: 5 animales Q95G96/Q95G96 (wt/wt), 3 animales S96/wt, 1 animal H95/wt y 1 animal H95/S96. Estos ciervos se encontraban en la fase terminal de la ECC

tras la inoculación oral de la cepa Wisc-1. Todos los animales mostraron un patrón de depósito de PrP^{CWD} similar en el SNC, aunque se observaron diferencias entre los distintos genotipos en el cerebelo, lo cual podría estar asociado a interacciones entre el agente infeccioso y factores propios del hospedador, entre otros su genotipo para el gen *PRNP*. Asimismo, el animal H95/S96 acumuló menores niveles de PrP^{CWD} en comparación con el resto de los genotipos en áreas rostrales del encéfalo, lo cual puede ser explicado por la existencia de un fenómeno de barrera de transmisión intraespecie o por ciertas diferencias en las propiedades de los priones acumulados por este animal. No obstante, el hallazgo más significativo de este estudio es que se observó que los ciervos que expresaban el alelo de resistencia H95 (los animales de los genotipos H95/wt y H95/S96) acumularon una cantidad no detectable, o significativamente menor de PrP^{CWD} , en varios tejidos periféricos, en comparación con los demás genotipos. Las mayores diferencias se observaron en páncreas, corazón, riñón e intestino. Además, se describe por primera vez el depósito de PrP^{CWD}, detectado mediante técnicas convencionales, en las arterias renales de cérvidos infectados por ECC. Por último, ninguno de los animales mostró acumulación de PrP^{CWD} en el músculo esquelético.

Estos cinco estudios han cumplido los objetivos descritos al inicio de la tesis, y contribuyen a conocer mejor los mecanismos reguladores de la barrera de transmisión para las EET, así como ciertos aspectos relacionados con su patogenia

Prion diseases have been described in numerous mammalian species. These diseases can have a spontaneous, familial, or acquired origin. However, certain species, including those of the Canidae family, appear to show a reduced susceptibility to these diseases. It is known that certain amino acid variants or PrP^{C} are main factors determining susceptibility to prion diseases. Previous studies using transgenic murine models revealed the importance of the N>D amino acid substitution, a key amino acid characteristic of canids, in the resistance to the transmission of several prion strains. The **first study** of this thesis was designed to evaluate whether the PrP carrying this substitution (N158D PrP) could act as a dominant-negative protein and prevent PrP^{Sc} formation when co-expressed with murine wild-type PrP^{C}. Transgenic mice co-expressing this mutant PrP showed a significant increase in survival times (by 45% to 113%) with respect to mice expressing similar levels of wild-type PrP^{C} after the inoculation of different prion strains (22L, ME7, RML, or 301C). Mice expressing the N158D PrP did not show significant differences in terms of the neuropathological or biochemical characteristics of the disease, indicating that the prolongation of the survival times observed in these animals was not due to strain modifications caused by the amino acid substitution of the dominant-negative protein. These results demonstrate that this specific substitution found in canids confers a dominant-negative effect on PrP, attenuating the conversion of PrP^{C} to PrP^{Sc} and delaying disease onset after the inoculation of prion strains of different characteristics.

Study number 2 represents a continuation of the first one. In this study, we evaluated the effect of the specific amino acid substitution of canids when introduced in the PrP^{C} of a species highly susceptible to a wide range of prion diseases. As a model of high susceptibility to TSE, transgenic mice overexpressing bank vole I109 PrP^{C} were selected. This PrP^{C} is so susceptible to misfolding that its only overexpression causes the development of a spontaneous TSE in these transgenic mice. Thus, we inoculated transgenic mice overexpressing bank vole I109 PrP and carrying this specific residue (TgVole-N159D mice), with two prion isolates of different characteristics the CWD-vole strain and an atypical strain of spontaneous origin. TgVole-N159D mice showed from 52 to 108% longer survival periods than TgVole mice controls with both inoculated strains. In addition, we observed that this residue was able to extend the survival period, not only in inoculated mice, but also in spontaneously sick animals. As observed in the first study, no differences were observed regarding the neuropathological features developed by both

transgenic models. These results indicate that the N>D amino acid substitution, characteristic of canids, exerts a protective effect against the propagation prions of very different characteristics even when expressed in a PrP^C highly susceptible to misfolding. The first two studies of this thesis indicate that the N> D amino acid substitution, characteristic of species poorly susceptible to TSE, could be a promising candidate in the development of gene therapeutic approaches against prion diseases.

After the BSE crisis and the appearance of vCJD in humans, mechanisms related to the transmission of TSE between species became a major public health concern. The implication of glycosylation of PrP^C in the conversion to its pathological form and in the interspecies transmission of prions has been extensively studied, although controversial results have been obtained. In the **study number 3**, we evaluate the effect of glycosylation of human PrP^C on the transmission barrier for prions and on the properties of different prion strains. Classical and atypical scrapie, CWD, L-BSE, H-BSE, classical C-BSE, ovine and porcine-passaged BSE, and vCJD isolates were used in *in vitro* and *in vivo* assays to analyze their propagation ability in a model expressing a non-glycosylated human PrP^C (transgenic line TgNN6h). Only BSE-related isolates (C- BSE, sheep BSE, porcine BSE, and vCJD) propagated in the non-glycosylated human substrate, suggesting the maintenance of the human transmission barrier for TSE. On bioassay, except for vCJD, the inoculation of the direct isolates did not cause disease in TgNN6h mice in a first passage. However, a very efficient transmission was achieved after PMCA adaptation of 3 BSE isolates (bovine, ovine and porcine) to the TgNN6h substrate and the subsequent inoculation of these PMCA-propagated isolates in TgNN6h mice. TgNN6h mice inoculated with PMCA-derived isolates or the vCJD direct isolate developed the characteristic neuropathological and biochemical features of BSE, suggesting that the absence of glycans in human PrP^C does not alter the pathobiological characteristics of BSE prions.

The mechanisms regulating the pathogenesis of TSE, especially those of the sporadic forms, remain largely unknown. Several studies indicate that the accumulation of misfolded proteins, not only in prion diseases, but in other neurodegenerative disorders, could induce endoplasmic reticulum stress and/or an impairment of protein degradation by the ubiquitin-proteasome system. For this reason, in **study number 4** two markers related to these processes were analyzed by immunohistochemical techniques, in brain samples from murine models of spontaneous TSE (TgVole mice). These mice also

expressed the Ub^{G76V}-GFP (TgU1 transgenic line), whose accumulation indicates a dysfunction of proteasomal degradation. Therefore, the accumulation of Ub^{G76V}-GFP was selected as a marker of the impairment of the ubiquitin-proteasome system, and the PDI protein as a marker of endoplasmic reticulum stress. $TgU1^{+}$/$TgVole^{+}$ mice of different ages were chosen to evaluate these pathogenic processes in the preclinical and clinical stages of spontaneous prion disease. As controls, $TgU1^{+}$/ $TgVole^{-}$ mice of similar ages were selected. $TgU1^{+}$/$TgVole^{+}$ clinical animals showed a significantly higher accumulation of both PDI and Ub^{G76V}-GFP than age-matched controls in certain brain areas. However, in $TgU1^{+}$/$TgVole^{+}$ preclinical mice no significant increase in endoplasmic reticulum stress or loss of proteasome function was detected in any of the brain regions analyzed. Therefore, none of these processes seems to be an early event in the pathogenesis of spontaneous forms of TSE. It was also observed that $TgU1^{+}$/$TgVole^{+}$ mice, especially those of the clinical group, showed an intense accumulation of Ub^{G76V}-GFP in reactive astrocytes. These preliminary results indicate that both endoplasmic reticulum stress and proteasome impairment are secondary mechanisms associated with the development of the neuropathological changes during spontaneous TSE, rather than key pathogenic mechanisms of these diseases.

Finally, in **study number 5**, we analyzed the immunohistochemical distribution pattern of PrP^{CWD} in the CNS, and in a wide range of peripheral tissues from ten white-tailed deer (*Odocoileus virginianus*) of different genotypes for the *Prnp* gene: 5 Q95G96/Q95G96 (wt/wt) animals, 3 S96/wt animals, 1 H95/wt animal and 1 H95/S96 animal. Deer were at the terminal stage of CWD after oral inoculation of the Wisc-1 strain. All animals showed a similar deposition pattern of PrP^{CWD} in the CNS, although differences were observed between the different genotypes in the cerebellum. These differences could be associated with interactions between the infectious agent and specific factors of the host, being the genotype for the *Prnp* gene one of these factors. In addition, the H95/S96 animal accumulated lower levels of PrP^{CWD} in rostral areas of the brain, in comparison with the rest of the genotypes. This lower accumulation may be explained by the existence of an intraspecies transmission phenomenon or by certain differential properties of the prions accumulated by this animal. However, the most relevant finding of this study is that deer expressing the H95 allele (animals of the H95/wt and H95/S96 genotypes) accumulated an undetectable, or a remarkably lower amount of PrP^{CWD} deposits in peripheral tissues, compared to the other genotypes. Most significant

differences were observed in pancreas, heart, kidney, and intestine samples. In addition, to our knowledge, this is the first description of PrP^{CWD} deposition, detected by conventional techniques, in renal arteries of CWD-infected deer. Finally, none of the animals showed accumulation of PrP^{CWD} in skeletal muscle samples.

These five studies have fulfilled the objectives described at the beginning of the thesis and contribute to a better understanding of the regulatory mechanisms of the transmission barrier for TSEs, as well as certain aspects related to their pathogenesis.

ANEXOS

Anexo 1. Protocolo de tinción con Hematoxilina-Eosina

Cortar las muestras de encéfalo con el micrótomo (4 micras) y recogerlas sobre portaobjetos.

-Desparafinado

Se realizan inmersiones de los tejidos en los siguientes reactivos:

1. Xilol: 4´
2. Xilol:4´
3. Alcohol 100º: 4´
4. Alcohol 100º: 4´
5. Alcohol 96º: 1´
6. Alcohol 70º: 1´
7. Agua corriente: 1´

-Tinción

Se realizan inmersiones de los tejidos en los siguientes reactivos:

1. Hematoxilina: 5´
2. Agua corriente: 1´
3. Alcohol ácido: 2´
4. Eosina: 2´
5. Agua: 3 inmersiones de 1-2´´

-Deshidratación y montaje:

1. Alcohol 100º: 1´
2. Alcohol 100º: 1´
3. Xilol: 1´
4. Xilol: mínimo 5´
5. Montaje de la muestra en Dibutylphtalate Poliestireno Xileno (DPX)

Anexo 2. Detección de la PrP^{Sc} mediante la técnica de Paraffin-embedded tissue (PET) blot

Cortar las muestras de encéfalo (4 micras) y colocarlas en una membrana de nitrocelulosa de 0.45 µm de Bio-Rad, dejar secar mínimo toda la noche en una estufa a 56º C

-Desparafinado

Exponer las membranas a los siguientes reactivos:

1. Tolueno: 2x5´
2. Isopropanol: 2x5´
3. Alcohol 95º : 2x5´
4. Alcohol 80º : 2x5´
5. Tween 20 al 0.1 % en agua destilada: 1x10´
6. Tween 20 al 0.05% en TBS (Tris buffered saline): 5´

-Digestión con Proteinasa K (PK)

1. Sumergir las membranas en una solución de pK a una concentración de 250 µg/ml en solución de TBS más 0.1% Brij 35P durante 2 h en baño maría de 55 a 56ºC
2. Lavar 10 ´en TBS + 0.05% Tween 20 en el agitador (rápido)

-Bloqueo

1. Realizar 3 lavados de 10´con TBS+ 0.05% Tween 20 con leche al 1%
2. Saturar la membrana con TBS+ 0.05% Tween 20 + 0.2% de BSA (Tampón de saturación) en agitación durante 30´

-Inmunodetección

1. Incubar 1 h con el anticuerpo monoclonal primario Sha31diluido en tampón de saturación (1:8000)
2. Lavar 3x10´con TBS+ 0.05% Tween 20 con leche al 1%
3. Incubar 30´con el anticuerpo secundario Dako Polyclonal Goat Anti-Mouse Inmunoglobulins/ AP, diluido en tampón de saturación (1: 500). Incubar en parafilm boca abajo
4. Lavar 5x10´con TBS+ 0.05% Tween 20 con leche al 1%

-Revelado

1. Ajustar las membranas a pH alcalino con TBS pH 9.5 + 50mmol/L de MgCl2, 2x 5´ en agitación rápida
2. Revelar con NBT/BCIP (Cloruro de nitroblue tetrazolium) en agitación lenta durante 45´

Anexo 3. Técnica inmunohistoquímica para la detección de PrP^{Sc} en encéfalo de ratón.

Cortar las secciones (3-5 μm) y recogerlas sobre portaobjetos tratados (starfrost).

Secar las secciones toda la noche a 56º C.

-Desparafinado:

1. Xilol: 5´
2. Xilol:5´
3. Alcohol 100º: 5´
4. Alcohol 100º: 5´
5. Alcohol 96º: 3´
6. Alcohol 70º: 3´

Pasar las preparaciones a agua destilada (H2Od).

No dejar secar las preparaciones a partir de este punto.

-Desenmascarado de epítopos:

1. Inmersión de las secciones en ácido fórmico (98%):10´
2. Lavar 10´en agua corriente.
3. Digestión con proteinasa K: 4g/ml, 15´a 37ºC ml de tampón precalentado.
4. Autoclavado hidratado con tampón citrato: 10´a 96ºC

-Inmunodetección

1. Cubrir las muestras con solución de peroxidasa (kit Envision-DAB. DAKO) durante 5´para bloquear la peroxidasa endógena.
2. Incubar las muestras con el anticuerpo primario anti-PrP durante 1 h (6H4, 1:100; 3F4 1:1000)
3. Lavar con Wash buffer (Tween 20 al 0.1 % en H2Od) 5´x3 veces.
4. Incubar con el polímero marcado Envision "goat anti-mouse" (kit Envision DAB, DAKO) durante 30´
5. Lavar con Wash buffer 5´x3 veces.
6. Incubar con el cromógeno DAB plus (kit Envision DAB, DAKO) 5´
7. Lavar con H2Od 5´x3 veces.
8. Contrateñir las muestras con hematoxilina durante 5´
9. Lavar con agua destilada

-Deshidratación y montaje

1. Alcohol 70º: inmersión (x 3), Alcohol 96º: inmersión (x 3), Alcohol 100º: inmersión (x3), Alcohol 100º: inmersión (x 3), Xilol: inmersión (x 3), Xilol: inmersión 5´
2. Montar en DPX

Anexo 4. Protocolo de Western Blot para la detección de PrP^{res} en encéfalo de ratón

Preparar un homogeneizado de encéfalo al 10% (peso/volumen) en PBS. Para ello se triturará la muestra utilizando tubos de trituración y un homogeneizador automático, realizando dos fases de homogeneizado a 6500 rpm

-Digestión

1. Añadir 10 µl de homogeneizado a 10 µl de una solución de sarcosyl (N-Lauroylsarcosine sodium salt) al 10%
2. Digerir con pK a una concentración de 80-170 µg/ml durante 1h a 37ºC
3. Parar la reacción con 20 µl de buffer de carga Laemmli (0.1% 2-Mercaptoethanol, 0.0005% Bromophenol blue, 10% Glycerol, 2% SDS, 63 mM Tris-HCl)
4. Incubar a 100ºC durante 5´

-Electroforesis

1. Preparar tampón de migración (50 ml tampón MES + 950 ml H2O destilada)
2. Cargar 20-25 µl en los pocillos del gel (Criterion XT 12% Bis-Tris Precast Gel)
3. Realizar la electroforesis a 80 V/30´ + 150 V/90´

-Transferencia

1. Preparar tampón de transferencia (1875 ml H2Od + 250 ml Tris CAPS 10 X + 375 ml etanol puro)
2. Activar (o equilibrar) membrana (Inmuno-Blot PVDF Membrane): poner membrana en cubetas (aclaradas con H2Od) con:
    a. Etanol puro: 15´´
    b. H2Od 5´ (en agitación lenta)
    c. Tampón de transferencia: 5´
3. Sacar el gel con cuidado manejándolo con una espátula húmeda
4. Realizar la transferencia a 150V durante 90´

-Fijación de proteínas

1. Poner la membrana en agitación suave en los siguientes reactivos:
    a. PBS 1x: 5-10´´
    b. Etanol puro: 5-10´´
    c. H2Od: 5´

-Inmunodetección

1. Bloquear la membrana en una solución de leche desnatada al 5% durante 60´
2. Lavar con PBST
3. Incubar la membrana con el anticuerpo primario anti-PrP durante 1 h (Sha31, 1:10000; 3F4, 1:10000; SAF83 1:400; 5C6 1:2000) diluido en PBST
4. Lavar con PBST 3x5´
5. Incubar con anticuerpo secundario conjugado con estreptavidina (1:5000) diluido en PBST durante 1h
6. Lavar con PBST 1x10´y 3x5´

-Revelado

1. Revelar durante 2´utilizando la solución de revelado (SuperSignal West Pico, Thermofisher)

Anexo 5. Técnica de inmunohistoquímica para la detección de PrP^C

Mantener las muestras a 60ºC un mínimo de 30 minutos antes de empezar la técnica.

-Desparafinado:
1. Xilol: 5´
2. Xilol:5´
3. Alcohol 100º: 5´
4. Alcohol 100º: 5´
5. Alcohol 96º: 3´
6. Alcohol 70º: 3´

Lavar 2x5 minutos con H2Od

-Desenmascarado de epítopos e inmunodetección

1. Bloquear la peroxidasa endógena utilizando la solución de bloqueo (kit Envision-DAB. DAKO, Dinamarca) durante 20´
2. Lavar con Wash buffer (Tween 20 al 0.1 % en H2Od) 5´x3 veces.
3. Autoclavado hidratado con tampón citrato: 30´a 100ºC
4. Incubar con anticuerpo primario anti-PrP (SAF32, 1:1000; 5C6, 1:1000) toda la noche a 4ºC. Controles negativos sin anticuerpo, sólo diluyente.
5. Dejar que las muestras alcancen la temperatura ambiente durante 30´.
6. Lavar con Wash buffer 5´x3 veces.
7. Incubar con el polímero marcado Envision “goat anti-mouse” (kit Envision DAB, DAKO) durante 30´

8. Lavar con Wash buffer 5´x3 veces.
9. Incubar con el cromógeno DAB plus (kit Envision DAB, DAKO) 5´
10. Lavar con H2Od 5´x3 veces.
11. Contrateñir las muestras con hematoxilina durante 5´
12. Lavar con agua destilada

-Deshidratación y montaje

1. Alcohol 70º: inmersión (x 3), Alcohol 96º: inmersión (x 3), Alcohol 100º: inmersión (x3), Alcohol 100º: inmersión (x 3), Xilol: inmersión (x 3), Xilol: inmersión 5´
2. Montar en DPX

Anexo 6. Técnica de tinción de Rojo Congo

Elaboración de las soluciones de trabajo**:**

A) Solución Madre I (de preincubación)
   a. 100 ml de alcohol de 80º+3g de NaCl
   b. Reposo de 24h
B) Solución de trabajo de preincubación
   a. 100 ml de Solución Madre I+1 ml de una solución de NaOH en agua (1%)
C) Solución Madre II
   a. 100 ml de alcohol de 80º +3g de NaCl + 0,5g de Rojo Congo
   b. Reposo de 24h
D) Solución de trabajo de tinción (Rojo Congo)
   a. 100 ml de Solución Madre II+1 ml de solución acuosa de NaOH al 1%

Técnica

-Desparafinado:

1. Xilol: 10´
2. Xilol:10´
3. Alcohol 100º: 5´
4. Alcohol 100º: 5´
5. Alcohol 96º: 5´
6. Alcohol 70º: 5´

Lavar 2x5 minutos con H2Od

-Tinción:

1. Teñir las muestras con hematoxilina durante 20´
2. Lavar en agua corriente 10´
3. Incubar con la Solución de trabajo de preincubación durante 20´
4. Incubar con la Solución de trabajo de tinción durante 20´

-Deshidratación y montaje

3. Alcohol 100º: inmersión (x 2)
4. Xilol: inmersión (x 3)
5. Xilol: inmersión 5´
6. Montar en DPX

Anexo 7. Técnica de inmunohistoquímica para la detección de GFAP

Mantener las muestras a 60ºC un mínimo de 30 minutos antes de empezar la técnica.

-Desparafinado:

1. Xilol: 5´
2. Xilol:5´
3. Alcohol 100º: 5´
4. Alcohol 100º: 5´
5. Alcohol 96º: 3´
6. Alcohol 70º: 3´

Lavar 2x5 minutos con H2Od

-Inmunodetección

1. Bloquear la peroxidasa endógena utilizando la solución de bloqueo (kit Envision-DAB. DAKO, Dinamarca) durante 5´
2. Lavar 3 veces con TBST
3. Incubar con anticuerpo primario anti-GFAP (DAKO, Dinamarca) 1:500 durante 30´
4. Lavar 3 veces con TBST
5. Incubar con el polímero marcado Envision “goat anti-rabbit” (kit Envision DAB, DAKO) durante 30´
6. Lavar 3 veces con TBST
7. Teñir las muestras con DAB durante unos minutos
8. Parar la reacción con agua destilada

9. Contrateñir con hematoxilina 5´ y lavar con agua destilada

-Deshidratación y montaje

1. Alcohol 70º: inmersión (x 3), Alcohol 96º: inmersión (x 3), Alcohol 100º: inmersión (x3), Alcohol 100º: inmersión (x 3), Xilol: inmersión (x 3), Xilol: inmersión 5´
2. Montar en DPX

Anexo 8. Técnica de inmunohistoquímica para la detección de la GFP

Mantener las muestras a 60ºC un mínimo de 30 minutos antes de empezar la técnica.

-Desparafinado:

1. Xilol: 5´
2. Xilol:5´
3. Alcohol 100º: 5´
4. Alcohol 100º: 5´
5. Alcohol 96º: 3´
6. Alcohol 70º: 3´

Lavar 2x5 minutos con H2Od

-Inmunodetección

1. Bloquear la peroxidasa endógena utilizando la solución de bloqueo (kit Envision-DAB. DAKO, Dinamarca) durante 30´
2. Lavar 3 veces con agua destilada y una en 1X PBST
3. Bloquear sitios de unión inespecíficos con suero de cabra al 10% en PBS durante 30 minutos
4. Incubar con anticuerpo primario anti-GFP (Abcam, Reino Unido) 1:2500 toda la noche a 4ºC. Controles negativos sin anticuerpo, sólo diluyente.
5. Dejar que las muestras alcancen la temperatura ambiente durante 30´.
6. Lavar las muestras 5 veces con 1XPBST (5´cada lavado)
7. Incubar con el anticuerpo policlonal goat anti-rabbit biotynilated (DAKO) 1:500 durante 30´
8. Lavar 5 veces con 1XPBST (5´cada lavado)
9. Incubar con complejo avidina-biotina (ABC kit, Vector Laboratories) durante 30´.

10. Lavar 4 veces con 1XPBST y 2 con agua destilada
11. Teñir las muestras con DAB durante unos minutos
12. Parar la reacción con agua destilada
13. Contrateñir con hematoxilina 5´ y lavar con agua del grifo
14. Deshidratar en alcohol de 96º (3 inmersiones) y de 100º (2 inmersiones)
15. Introducir las muestras en xilol y montar en DPX

Anexo 9. Técnica de inmunofluorescencia para la detección de la GFP y GFAP

Mantener las muestras a 60ºC toda la noche antes de empezar la técnica.

-Desparafinado:

1. Xilol: 5´
2. Xilol:5´
3. Alcohol 100º: 5´
4. Alcohol 100º: 5´
5. Alcohol 96º: 3´
6. Alcohol 70º: 3´

Lavar 2x5 minutos con H2Od

-Desenmascarado de epítopos e inmunodetección

1. Bloquear la peroxidasa endógena con H2O2 al 1% durante 30´
2. Lavar con PBS 5´x 3 veces
3. Pretratar las muestras con 0,1% Triton X-100 durante 3 h a temperatura ambiente
4. Autoclavado hidratado con tampón citrato: 10´a 121ºC
5. Lavar con PBS 5´x 3 veces
6. Incubar con anticuerpo primario anti-GFP (Abcam, Reino Unido) y anti-GFAP (1:200; Dako) 1:2500 toda la noche a 4ºC
7. Dejar que las muestras alcancen la temperatura ambiente durante 30´
8. Lavar con PBS 5´x 3 veces
9. Incubar con el anticuerpo goat anti-mouse IgG biotin conjugate (1:100; Invitrogen) y Alexa fluor 594 streptavidin conjugate (1:1000; Invitrogen) durante 1 h en oscuridad

-Deshidratación y montaje

10. Deshidratar en alcohol de 96º (3 inmersiones) y de 100º (2 inmersiones)
11. Introducir las muestras en xilol y montar en medio acuoso

Anexo 10. Técnica de inmunohistoquímica para la detección de la proteína PDI

Mantener las muestras a 60ºC un mínimo de 30 minutos antes de empezar la técnica.

-Desparafinado:

8. Xilol: 5´
9. Xilol:5´
10. Alcohol 100º: 5´
11. Alcohol 100º: 5´
12. Alcohol 96º: 3´
13. Alcohol 70º: 3´

Lavar 2x5 minutos con H2Od

-Desenmascarado de epítopos:

5. Autoclavado hidratado con tampón citrato: 10´a 96ºC

-Inmunodetección

1. Bloquear la peroxidasa endógena utilizando la solución de bloqueo (kit Envision-DAB. DAKO, Dinamarca) durante 5´
2. Lavar 3 veces con TBST
   Incubar con anticuerpo primario anti-PDI (Santa Cruz Biotechnology) 1:200 durante 1h.
3. Lavar 3 veces con TBST
4. Incubar con el polímero marcado Envision "goat anti-mouse" (kit Envision DAB, DAKO) durante 30´
5. Lavar 3 veces con TBST
6. Teñir las muestras con DAB durante unos minutos
7. Parar la reacción con agua destilada
8. Contrateñir con hematoxilina 5´ y lavar con agua destilada
9. Lavar con agua destilada

-Deshidratación y montaje

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