# UNIVERSIDAD COMPLUTENSE DE MADRID

## FACULTAD DE VETERINARIA

### DEPARTAMENTO DE SANIDAD ANIMAL



# TESIS DOCTORAL

## ROLE OF POLYMORPHIC VARIANTS OF THE PRION PROTEIN ON THE RESISTANCE-SUSCEPTIBILITY TO PRION INFECTION

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR


**Patricia Aguilar Calvo**

Directores

Juan María Torres Trillo
Juan Carlos Espinosa Martín


**Madrid, 2014**

**UNIVERSIDAD COMPLUTENSE DE MADRID**

**FACULTAD DE VETERINARIA**

Departamento de Sanidad Animal


# ROLE OF POLYMORPHIC VARIANTS OF THE PRION PROTEIN ON THE RESISTANCE/SUSCEPTIBILITY TO PRION INFECTION

**Patricia Aguilar Calvo**

TESIS DOCTORAL

Dirigida por

Dr. Juan María Torres Trillo

Dr. Juan Carlos Espinosa Martín

**UNIVERSIDAD COMPLUTENSE DE MADRID**
**FACULTAD DE VETERINARIA**
**Departamento de Sanidad Animal**



# ROLE OF POLYMORPHIC VARIANTS OF THE PRION PROTEIN ON THE RESISTANCE/SUSCEPTIBILITY TO PRION INFECTION

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

**Patricia Aguilar Calvo**

Madrid, 2014

DIRECTORES:
**Dr. Juan María Torres Trillo**
**Dr. Juan Carlos Espinosa Martín**

Los doctores Juan María Torres Trillo y Juan Carlos Espinosa Martín, investigadores titulares en el Centro de Investigación en Sanidad Animal (INIA-CISA) HACEN CONSTAR:

1) Que la Tesis presentada por Patricia Aguilar Calvo titulada "**Role of polymorphic variants of the prion protein on the resistance/susceptibility to prion infection**", ha sido realizada bajo nuestra dirección y que, tras su revisión, consideramos que tiene la debida calidad para su presentación y defensa.

Y para que así conste, lo firmamos en Madrid a ocho de octubre de dos mil catorce.

**Fdo: Dr. Juan María Torres Trillo** **Fdo: Dr. Juan Carlos Espinosa Martín**

**EUROPEAN DOCTORATE MENTION**

This thesis has been proposed for the European doctorate mention by virtue of the following European research stays and thesis reports:

Research stays:

- École Nationale Vétérinaire de Toulouse, France. Dr. Olivier Andréoletti´s laboratory; 3 months in 2013.
- Institut National de la Recherche Agronomique (INRA), Jouy-en-Josas, Paris, France. Dr. Vincent Béringue´s laboratory; 3 months in 2012.

Thesis reports:

- Dr. Human Rezaei. Institut National de la Recherche Agronomique (INRA), Jouy-en-Josas, Paris, France.
- Dr. Guiseppe Legname. Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste, Italy.
- Dr. Esperanza Gómez-de Lucía Duato. Universidad Complutense de Madrid (UCM), Spain.

## AGRADECIMIENTOS

Ahí va, el último apartado que escribo pero el primero por su relevancia. En la tesis al final sólo he puesto experimentos, muuuuchas tablas y abundante blablablá en la discusión pero en realidad, detrás de todo eso, hay mucho más. Hay cuatro años de grandes experiencias con gente fantástica. Por ello, me gustaría empezar este libro dedicando unas palabras a todas las personas que lo han hecho posible.

Ahí va, el último apartado que escribo pero el primero por su relevancia. En la tesis al final sólo he puesto experimentos, muuuuchas tablas y abundante blablablá en la discusión pero en realidad, detrás de todo eso, hay mucho más. Hay cuatro años de grandes experiencias con gente fantástica. Por ello, me gustaría empezar este libro dedicando unas palabras a todas las personas que lo han hecho posible.

En primer lugar quiero dar las GRACIAS con mayúsculas a los responsables de todo esto: mis directores de tesis **Juanma** y **Juan Carlos**. No sé cómo agradeceros la oportunidad que me habéis dado. Esta tesis marca un antes y un después en mi vida. No soy la misma persona que llegó hace cuatro años en el CISA. Esa ingenua veterinaria que no sabía nada de bioquímica y poco de la vida. Esta tesis comprende los mejores años de mi vida y todo ello comenzó por vosotros.

**Juanma**, gracias por tu exigencia. Por tu "ocúpate y deja de preocuparte", por tu "puedes hacerlo mejor". Porque has creído en mí cuando ni tan siquiera yo lo hacía. Porque me has demostrado tantas veces que podía y has peleado porque no hundiera el barco (tú no lo sabes pero tus discursos de motivación son los más bonitos que nadie me ha dedicado nunca). Gracias por nuestras batallas dialécticas que aunque agotadoras, siempre me han encantado. Sé que soy una privilegiada porque me has brindado todas las oportunidades con las que contabas y muchas más. Gracias de corazón. Sólo espero haber estado a la

altura y que como yo puedas hacer el balance más positivo posible de nuestros años juntos.

A **Juan Carlos**; ay cómo te voy a echar de menos. Sé que eres mi jefe pero también un gran amigo. Me has escuchado, has sido salvavidas en mis mayores lloreras, me has regañado cual padre, me has aconsejado cual padre, has estado ahí siempre. Me llevo mucho de ti, tanto personal como profesional. Tanto, tanto que me he sorprendido muchas veces andando por los pasillos con las manos en la espalda o diciéndole a la gente "vayamos por partes, como diría Jack el destripador". Me consuela saber que tú contestas el teléfono diferente desde que me conoces; ¿a que no te habías dado cuenta? Es sorprendente lo que se puede llegar a aprender de las personas… Ojalá se me hubiera pegado un poquito más de tu serenidad. ¡Muchas gracias Juan Carlos!

Al **grupo de priones**. A mi **Irene**, ¡qué corazón más grande tienes! Eres la bondad personificada. No sabes lo mucho que me han ayudado nuestras charlas, tus intentos de abrazo (lo siento, a mí eso no me va mucho), tus "bequi, ¿qué tal el fin de semana?". Es genial cómo te preocupas por las personas. Gracias por cuidarme. A **Juan**, por tu paciencia, tu sonrisa adornando el "buenos días becaria". Admiro tu profesionalidad y las ganas que pones en todo lo que haces. Siento no haberte acompañado de pesca, eso queda pendiente. No te olvides, por favor. A **Patri**, mi tocaya. Siempre liada con los Westerns, muchos míos... Me has salvado tantas veces que no hay hojas para darte las gracias. A Ana, **la Esteban**, por dedicarme tiempo para enseñarme. Haces que todo parezca más fácil y divertido, ¡incluso los kits! A **Ana Villa**, la bióloga seria. Me costó sacarte una sonrisa pero la he visto y me encanta. Eres muy buena profesora, de verdad que te agradezco tus cursos rápidos de genética y química. Los echaré mucho de menos. Gracias a los que estuvieron pero ya se fueron. Todos sois parte de esta tesis; gracias por hacerla posible.

A **Belén Pintado**, del CNB, por las microinyecciones que han dado lugar a tooooodas las líneas `de ratón transgénico de este estudio.

A **los de peces**. Ese maravilloso grupo que creció y creció y un día apareció sin avisar en nuestro laboratorio. Gracias por los cafés, los chistes, las respuestas rápidas a mis más absurdas dudas. A mi **Carol**, que seguro que se está buscando como una loca entre estas líneas. Si es que no me crees cuando te digo que eres mucho más que una amiga CISA para mí. Hemos compartido sufrimiento, desesperación de becaria, marujeos. Eso une más de lo que tú te piensas. Me tienes para lo que quieras.

A **la familia CISA**. A Tony porque transmites alegría a raudales; siempre con una sonrisa. A Nazaret, sus artículos y sus reflexiones. A Fernando y a Diana últimas pero valiosas adquisiciones de esta gran familia. A Lillian por tu "buen día" de todas las mañanas. A todos las personas con las que me cruzaba por los pasillos todos los días. Gracias a todos por esos pequeños detalles que hacen la vida ATeresita, nos vemos pronto.

A **Vincent,** no puedo más que darte las gracias por todo tu apoyo, por tu acogida, tu ayuda y tus enseñanzas. Y por el grupo tan genial que tienes. Carola, Guillaume, Human, Iman, Michell, Danicsa.. ¡todos! Pero especialmente a Mohammed. Te llevo en el corazón ☺

A **Olivier**, por tu peculiar forma de ver la vida. Me gusta. Gracias por los PET-blots y las cervezas, las histologías y los vinos, las PMCAs y el ibuprofeno. Aprendí mucho en Toulouse, ¡muchas gracias!

A **Joaquín** y los priónicos de Bilbao; gracias por hacerme un hueco en vuestro grupo. Gracias a Mayela y a Hasier por acogerme en vuestra casa. A los priónicos madrileños, maños, gallegos, catalanes. Me encanta reencontrarme con vosotros en los congresos. Os echaré de menos.

A **mi familia**. Gracias por tanto. Sé que todo lo bueno que pueda tener es gracias a vosotros y lo malo, seguramente lo aprendí mientras no mirabais. A mi padre, eres mi modelo. Todo te lo pregunto y todo lo sabes. Tus consejos son los mejores y recurro a ti cuando me ladra la conciencia; me guías. A mi madre que tanto se ha preocupado por mí. Sé que has sufrido viéndome tan estresada

pero, ¿has visto?, al final tendré recompensa. Mamu, gracias por tu alegría y tu gran corazón; eres el mejor de los apoyos. A mi hermana, mi mejor amiga. Siempre estás ahí, la primera, confiando en mí, apoyándome y ahora dándome el mejor regalo que nunca me hayan hecho. Intentaré ser buena tía, aunque está el listón muy alto, ¿verdad tía Blanca? Muchas gracias tía, sabia como tú sola. Siempre encuentras el refrán que responde a mis dilemas y el ejemplo que clarifica hasta el problema más complejo. **Muchas gracias familia por los domingos.**

A mis **taquis**. Alfa, GG, Mery, Celi, Cris, Ame, Arro, Shey, vaya grupo de amigas tengo. Gracias por apoyarme siempre y no olvidarme. He vivido tantas cosas buenas con vosotras que me cuesta hacerme a la idea de que os vaya a tener lejos. Sois las mejores.

A **David**, por todo lo que me has ayudado siempre; nunca voy a olvidarlo. A **Pastorcito, Pauli** y **Pris**. Empezamos juntos nuestras de andanzas y sufrimiento de becarios. Gracias por nuestras reuniones para ahogar penas. Los experimentos fallidos parecen menos malos en el bar, ¿verdad? Gracias a Saluvet, el primer grupo de investigación que me acogió. Aun no entiendo cómo confiasteis en mi loca yo.

A **Jaime.** Espero que no te de vergüenza pero quería dedicarte unas palabras. Gracias por tu paciencia, por aguantar mis locuras, mis lloros, mis cambios de humor, los madrugones. Gracias por los viajes-congresos, los viernes de escapada, los paseos por el Retiro, los viajes diarios al CISA, tus visitas a Francia, la feria, Cerdeña. Gracias por descubrirme tantas cosas y hacer especial lo más mundano. Qué suerte la mía de cruzarme contigo en el autobús del CISA. Desde entonces has estado ahí apoyándome, tranquilizándome, haciéndome especial. Eres el mejor compañero que se puda pedir. **¡Bendito CISA, cuánto me has dado!**

Por último quería darle las gracias a Christina Sigurdson por creer en mí y darme una oportunidad en su laboratorio. ¡No te decepcionaré!

**AGRADECIMIENTOS**

Y esto es todo amigos. Mis días en el CISA están llegando a su fin. Estos cuatro años con todos vosotros han dado mucho de sí. He tenido tiempo para decepcionarme y emocionarme, luchar y rendirme, llorar y reir, desenamorarme y enamorarme, desaprender y aprender, preocuparme y ocuparme, irme y volver y mucho, mucho más. Esta tesis ha sido una gran experiencia profesional pero sobre todo personal. Creo que algo he madurado y aprendido más. Pese a los agobios o malos momentos, hoy puedo concluir: **Ay la tesis, ¡qué gran invento!**

## ACKNOWLEDGEMENTS

And this it is, the end; the final part of this book of so many adventures. In the end, I decided just to include a few experiments, loads of tables and a lot of words in the discussion. However, there´s so much more behind all of this — four long years of fascinating experiences and wonderful people. Please bear with me a while and allow me to dedicate you all a few words.

First of all, I would like to say a huge 'thank you' to the two directors of my thesis, Juanma and Juan Carlos. I cannot thank you enough for the opportunity you have given me. This thesis marks a veritable milestone in my life. Today, I´m not the same person as that innocent vet who arrived in CISA four years ago, knowing nothing about chemistry and very little about life. This thesis represents the best years of my life and it all started with you.

**Thank you Juanma** for your thoroughness — for your "don´t worry, just get on with it" and your "you can do it better"; for believing in me when I didn't; for showing me that I could and stopping me from sinking. You should know that your motivational speeches are the best that I have ever heard; thank you too for our dialectic battles that, although exhausting, I really loved. I know I'm lucky because you gave me the best opportunities you could — and more. I thank you from my heart. I hope I lived up to your expectations and that the balance of these four years together has been positive for you too.

**Thank you Juan Carlos** — you don´t know how much I´m going to miss you! You are my boss but my friend too. You have listened to me, helped me in the bad moments, scolded me like a father, advised me like a father; you were always there. I have taken a lot from you in both a personal and a professional sense. And I hope you have taken something from me too. It´s surprising how much we can learn from other people. Thank you so much Juan Carlos!

To **the prion group**. To my beloved Irene — what a huge heart you have. You are goodness personified. I don't think you realize just how much you helped me with our talks, your attempts to hug me (I´m not very keen on these things, sorry!), your "how was the weekend?". It´s truly wonderful how you take care

of people. Thanks for worrying so much. Thank you Juan for your patience, your smiles when wishing me a good day. I admire your professionalism and your enthusiasm for everything. I´m so sorry that we finally didn't get to go fishing — it'll have to wait. But, please, don't forget. To Patri, always busy with your Western blots, and often with mine! There is not enough room here to express my gratitude to you. To Ana Esteban, for taking time to teach me. You made things so easy, and funny … To Ana Villa, the serious biologist. It was difficult to make you smile at first, but I did it in the end and I loved it!!! Thank you for your quick masters course in chemistry and genetics — I will really miss them. I would also like to thank everybody that once belonged to our group but who has now left. You are also part of this thesis and thank you all for making it possible.

To **Belén Pintado** at CNB, for the embryo microinjections that gave rise to aaaaaaaaaaaaall the mouse lines in the study.

To the **fish group** (sounds horrible in English…). This wonderful group that started growing and then one day appeared by surprise in our lab. Thank you for the coffee, the jokes, the gossip, the quick answers to stupid questions. Thank you Carol! I´m sure that you will be looking like mad for your name in these lines. You don´t believe me when I say that you are more than a CISA friend. We suffered together so many desperate grad-student moments. That has meant a lot to me. You can count on me for anything you need.

To the **CISA family**. Thank you Tony for your happiness and your smiles. Thank you Nazaret for your articles and your reflections. And, last but not least, thank you Fernando and Diana, two final members of this huge family. There are so many people I should thank for the tiny things that made everything possible. Thank you CISA family!

Thank you **Vincent**, for your unconditional support, your help, your lessons. Thank you for having such a great group of people: Guillaume, Human, Iman,

Michell, Danicsa, and everybody at INRA. Thank you again, you are amazing! And, above all Mohammed, You are in my heart ☺.

Thank you **Olivier** for your very own way of thinking —I love it; for your PET-blots and beers, histologies and wine, PMCAs and aspirins. I learnt so much from you. Thank you so much!

Thank you **Joaquín** and prionics from Bilbao for making space for me in your group. Thank you Mayela and Hasier for making me welcome in your house. Thank you to prionics from Madrid, Zaragoza, Barcelona, Santiago de Compostela, etc. It's great bumping into you at conferences. I will miss you.

Thank you too so much to **my family**. I know that all the good things I have are thanks to you — and that I probably learnt the bad things when you weren't looking! Thank you father, you are my model. Whenever I ask you anything, you always know the answer. You have given me the best advice, you have guided me. Thank you mother for worrying so much about me. I know you suffered when you saw that I was so tired and stressed — but look! I did it! I would not have been able to do it without you. Thank you to my sister, my best friend. You have always been there for me. You were the first to believe in me, to support me and now you are going to give me the best gift of all. I´ll try to be a good aunt, I promise. However, the standard is high, isn´t it Aunt Blanca? — so wise as only you can be. Thank you Aunt for your wise sayings that always solved my troubles. **Thank you family for Sundays.**

Thank you friends: Alfa, GG, Mery, Celi, Cris, Ame, Arro, Shey — what a wonderful group of friends! I´ve lived so much with you that I cannot imagine leaving you. You are the best!

Thank you **David**, for all the help you gave me. I will never forget it. Thank you **Pastorcito, Pauli,** and **Pris** too. We all started on our way in science together. We have cried together and laughed about our failings. Problems don´t look so bad in a bar, do they? Thank you very much! Thanks too to **Saluvet**, my first research group. Thank you for trusting young foolish me.

**ACKNOWLEDGEMENTS**

Thank you **Jaime**. I hope that this doesn´t make you blush too much but I would like to dedicate you some special words. I would like to thank you for your patience, for putting up with my madness, my tears, my outbursts, the early mornings. Thank you for our journeys to congresses, our walks through El Retiro, our daily journeys to CISA, our Friday trips, your visits to France, the fair, Sardinia, and so on. Thank you for showing me so many things and making special things seem normal. How lucky I was to meet you on the bus. I have relied on you since then. You are the best partner I could have had. CISA has given me so much more than I expected!

Finally, I would like to thank **Christina Sigurdson** for trusting me and giving me such a wonderful opportunity to work in her laboratory. I won´t disappoint you!

And that´s all folks! My days at CISA are coming to the end. These four years have surpassed my expectations. I´ve had time to let myself go, to fight and surrender, to cry and smile, to fall out of love and back in love, to forget and to learn, to worry and to work, to leave and come back, and so much more. This thesis has been a fantastic professional experience but, even more, it has been a personal journey. I´ve grown up and learned so much. And, despite the bad moments, I can't help but conclude — **theses are such a wonderful invention!**

A Sarah, ven pronto.

(To Sarah, I cannot wait to see you)

**"Passion makes idiots of the cleverest men,**

**and makes the biggest idiots clever."**

**Máximas, F. de la Rochefoucauld (1665)**

# INDEX

**LIST OF FIGURES**

## LIST OF TABLES

## GLOSSARY

µl: microliter
µm: micrometer
129/Ola: wild type mouse line
12B2: anti-PrP monoclonal antibody (Yull et al., 2006)
9A2: anti-PrP monoclonal antibody (Yull et al., 2006)
+/+: homozygous
+/-: hemizygous
aa: aminoacid
B6CBAF1: mouse line
BoPrP-Tg110: transgenic mouse line expressing bovine PrP
BSA: bovine serum albumin
BSE: bovine spongiform encephalopathy
CWD: chronic wasting disease
dpi.: days post inoculation
DNA: deoxyribonucleic acid
ECL: western blotting detection reagents
EDTA: ethylenediaminetetraacetic acid
FH11: anti-PrP monoclonal antibody (Foster et al., 1996b)
g: gram
*g:* relative centrifugal force
GPI: glycosyl phosphatidyl inositol
GSS: Gerstmann-Sträussler-Scheinker disease
h: hour
HE: Hematoxylin-Eosin staining
IC: intracerebral
$ID_{50}$: Infectious Dose 50
IFF: insomnia familiar fatal
IgG: immunoglobulin G
IHC: Immunohistochemistry
K: kilodaltons
KO: knock out
LRS: lymphorethicular system
M: meter
mA: milliamper
mAb: monoclonal antibody
mg: milligram
min.: minutes
ml: milliliter
mm: millimeter
mM: millimolar
mpi.: months post inoculation
ng: nanogram
nt: nucleotide
O/N: over night
$^{o}$C: Celsius degree
ORF: Open Reading Frame
PBS: phosphate buffered saline
PBST: phosphate buffered saline with tween
PCR: polymerase chain reaction
PET blot: paraffin-embedded tissue blot

PK: proteinase K

PMCA: Protein Misfolding Cyclic Amplification

Prion: acronym of proteinaceous infectious particle only

*prnp:* gene that codes for the prion protein

PrP: prion protein

$PrP^{27-30}$: protease-resistant core of $PrP^{Sc}$

$PrP^{c}$: cellular prion protein isoform

$PrP^{res}$: protease-resistant core of $PrP^{Sc}$

$PrP^{Sc}$: scrapie prion protein isoform (disease associated isoform)

s: seconds

Sc: scrapie

SDS: sodium dodecyl sulphate

SEM: standard error of the mean

Sha31: anti-PrP monoclonal antibody (Feraudet et al., 2005)

Tg: Transgenic

TBS: tris buffered saline

TBST: tris buffered saline with tween

TE: loading buffer (10mM Tris, pH 7.4, 0.1mM EDTA)

TSE: Transmissible Spongiform Encephalopathy

V: volt

Wt: wild type

w/v: weight/volume

WB: Western blot

# I. RESUMEN

Introducción

Las Encefalopatías Espongiformes Transmisibles (EETs) son enfermedades neurodegenerativas progresivas y mortales que afectan a los seres humanos y a otras especies de mamíferos e incluyen la enfermedad de Creutzfeldt-Jacob (ECJ), la enfermedad de Gerstmann-Sträussler-Scheinker (GSS) y el insomnio familiar fatal (FFI) en los seres humanos, la tembladera en ovinos y caprinos, la encefalopatía espongiforme bovina (EEB) en el vaca, y la caquexia crónica (CC) en ciervos y alces.

Un acontecimiento fundamental en la patogénesis de las EETs es el cambio conformacional de la proteína priónica celular ($PrP^{C}$) en una proteína priónica patógena ($PrP^{Sc}$) con plegamiento aberrante que es capaz de propagarse mediante la conversión de más $PrP^{C}$ en $PrP^{Sc}$. Es bien sabido que ciertos cambios aminoacídicos en la secuencia de la $PrP^{C}$ pueden afectar a la eficiencia de conversión de la $PrP^{C}$ y a la transmisión de priones. De esta forma, individuos de una especie pueden presentar diferentes niveles de susceptibilidad a un agente priónico dependiendo de su genotipo de la PrP. Este hecho tiene importantes implicaciones en el diseño de estrategias encaminadas a controlar y erradicar las EETs a través del cruzamiento selectivo de los individuos portadores de genotipos de la PrP asociados con resistencia a la infección con priones. Así, los programas de cría selectiva aplicados en el ganado ovino para promover el genotipo $A_{136}R_{154}R_{171}$, con resistencia conocida a la tembladera clásica, han reducido notablemente la prevalencia de esta enfermedad en muchos países europeos.

Aparte de las diferencias en la secuencia aminoacídica entre la $PrP^{C}$ y la $PrP^{Sc}$, las cepas de priones también modulan la conversión de la $PrP^{C}$. Por ejemplo, las ovejas que expresan el genotipo $A_{136}R_{154}R_{171}$ tienen una mayor susceptibilidad a la tembladera atípica que las ovejas que expresan otros genotipos como el VRQ. Por el contrario, las ovejas ARR son menos susceptibles a la tembladera clásica que las ovejas VRQ. Este hecho pone en duda los logros de los programas de cría selectiva cuyo objetivo es prevenir la infección de las ovejas con tembladera

clásica. Todos estos datos sugieren que la infección con priones estaría modulada por una combinación compleja de las propiedades conformacionales de los agregados de $PrP^{Sc}$ y las estructuras locales de la $PrP^{C}$. Por tanto, es necesario estudiar los cambios en la secuencia de la PrP en combinación con las cepas de priones a fin de avanzar en el conocimiento de los mecanismos moleculares que regulan la susceptibilidad/resistencia a la infección con priones; información con implicaciones fundamentales en el control y erradicación de las EETs en distintas especies.

2. Objetivos y diseño experimental

El objetivo principal de esta tesis es mejorar nuestra comprensión de los mecanismos moleculares que gobiernan la susceptibilidad/resistencia a la infección con priones. Para ello, se llevó a cabo una serie de objetivos específicos:

1. Estudiar el papel individual de las variantes polimórficas que se encuentran naturalmente en el gen de la proteína priónica (*prnp*) de cabra y de oveja en la susceptibilidad/resistencia a la tembladera y la EEB en ratones transgénicos (Tg). Para este fin, hemos generado un panel de líneas de ratón Tg que expresan o bien la $PrP^{C}$ de la cabra de tipo salvaje (wt) o variantes de la $PrP^{C}$ debidas a cambios puntuales de aminoácidos ($I/M_{142}$, $R/H_{154}$, $R/Q_{211}$, $Q/K_{222}$, $Q/R_{171}$ y N/K176). Estas líneas de ratón Tg fueron desafiadas con varias cepas de priones y sus susceptibilidades se evaluaron y se compararon con el fin de determinar el papel individual de cada variante de $PrP^{C}$ en la resistencia/susceptibilidad a la infección con priones.

2. Confirmar en el huésped natural (cabras) la fiabilidad de los resultados de los modelos de ratón Tg. Para ello, se llevaron a cabo inoculaciones experimentales en cabras que expresaban diferentes polimorfismos de la *prnp* con un caso de campo de tembladera o con un aislado de EEB en cabra y sus resultados se compararon con los obtenidos en los ratones Tg.

3. Materiales y Métodos

Se generó una colección de líneas de ratón Tg que expresaban diferentes variantes polimórficas de la $PrP^C$ de cabra y de oveja ($M_{142}$, $H_{154}$, $R_{171}$, $K_{176}$, $K_{222}$ y $Q_{211}$) mediante mutagénesis dirigida del plásmido pMo-GoPrP.Xho utilizado para la obtención de la línea Wt-Tg501 que expresa la $PrP^C$ de cabra wt. Los fundadores fueron caracterizados i) mediante PCR para determinar su capacidad de transmisión del transgén mutado a la progenie; ii) mediante Western blot (WB) para analizar sus niveles de expresión de la $PrP^C$ en cerebro y iii) su estado de comportamiento y de salud. Las líneas de ratón Tg seleccionadas se inocularon por vía intracerebral (IC) en el lóbulo parietal derecho con una variedad de aislados (homogeneizados de cerebro infectados por priones) que diferían en la cepa (tembladera clásica o atípica, o EEB clásica o atípica), el origen geográfico, el genotipo de la $PrP^{Sc}$, el perfil de la $PrP^{res}$ por WB, y en su comportamiento al ser transmitidas en ratones Tg bovinos BoPrP-Tg110. Mediante el estudio comparativo de las características de transmisión de cada línea Tg (tiempo de supervivencia, tasas de ataque, perfil de la $PrP^{res}$ por WB, y alteraciones histopatológicas) determinamos la susceptibilidad diferencial a la infección con priones asociada a cada variante de la $PrP^C$.

En paralelo, trabajamos en colaboración con el ENV (Francia) y el FLI (Alemania) para llevar a cabo estudios de transmisión de priones en cabras. Por un lado, se desafiaron cabras que expresaban diferentes genotipos de la *prnp* (WT, $M_{142}$, $H_{154}$, $Q_{211}$ o $K_{222}$) con un caso natural de tembladera clásica por vía oral o IC. Para los desafíos orales, los cabritos recibieron una primera dosis del inóculo (cerebro infectado con tembladera clásica) por amamantamiento natural dentro de las primeras 48 horas después del nacimiento y una segunda dosis a la edad de 30 días. Para los desafíos IC, el inóculo (el mismo cerebro infectado con tembladera clásica) se inyectó en la corteza temporal de cabras de seis meses de edad. Ambos grupos de animales fueron monitorizados para el desarrollo de enfermedades neurológicas y sacrificados en la fase clínica de la enfermedad. La presencia de $PrP^{Sc}$ en sus cerebros se evaluó mediante WB e

inmunohistoquímica. Por otro lado, se inocularon cabras con genotipos de la *prnp* WT, $R/Q_{211}$ o $Q/K_{222}$ por vía oral con homogeneizado de cerebro de cabras infectados con EEB por amamantamiento natural. Las cabras fueron sacrificadas en diferentes momentos del período de incubación y después de la aparición de los signos clínicos, y sus cerebros y varios tejidos periféricos se analizaron para la detección de la $PrP^{Sc}$ por WB, la determinación de alteraciones histopatológicas y la detección de infectividad priónica mediante bioensayo en ratones BoPrP-Tg110.

4. Resultados y discusión

Todas las líneas de ratón Tg seleccionadas expresaron niveles de $PrP^{C}$ en sus cerebros similares a los de cabra de cabra. En cada caso, la $PrP^{C}$ expresada era idéntica a excepción de los cambios aminoacídicos puntuales correspondientes a las variantes polimórficas deseadas ($I/M_{142}$, $R/H_{154}$, $Q/R_{171}$, $N/K_{176}$, $R/Q_{211}$ y $Q/K_{222}$). Todas las líneas mostraron un comportamiento fisiológico normal y no se observó ninguna alteración histopatológica en su cerebro lo que nos permitió descartar el desarrollo de una enfermedad neurológica espontánea como resultado de la expresión de la $PrP^{C}$ mutada.

Los ratones Wt-Tg501, con niveles de expresión de la $PrP^{C}$ en el cerebro similares a los de cabra (1X), fueron susceptibles a todos los aislados de tembladera y EEB inoculados independientemente de las propiedades de los aislados mencionadas en el apartado anterior. Los ratones Wt-Tg501fueron capaces de replicar todos estos agentes de priones manteniendo sus propiedades biológicas y bioquímicas. Además, esta línea de ratón nos permitió identificar al menos cuatro cepas de tembladera clásica en el panel de aislados inoculados. Estos resultados demuestran que la línea Wt-Tg501 es un modelo muy valioso para el estudio de la susceptibilidad de las cabras y ovejas a diferentes cepas de la tembladera y la EEB, así como para identificar y caracterizar las cepas de priones que afectan a estos rumiantes.

Por el contrario, los ratones $K_{222}$-Tg516, con niveles de expresión 1X de la $PrP^{C}$ mutada $K_{222}$- $PrP^{C}$, fueron resistentes a la transmisión primaria de todos los

aislados de tembladera clásica inoculados así como a EEB clásico y EEB-L atípico de vaca, pero no contra la tembladera atípica o el EEB de oveja o cabra. Por lo tanto, el cambio aminoacídico glutamina-lisina en el codón 222 ($Q/K_{222}$) proporciona resistencia contra la infección con los agentes de tembladera clásica, EEB clásica y EEB-L atípica de vaca pero no contra la tembladera atípica o EEB adaptada a oveja o a cabra. Por otro lado, los ratones heterocigotos $Q/K_{222}$ fueron resistentes a la inoculación con varios aislados de tembladera clásica. Estos ratones heterocigotos sólo fueron susceptibles a algunos aislados de tembladera clásica mostrando menores tasas de ataque (TA) y tiempos de supervivencia (TS) que los ratones Wt-Tg501 lo que indica que la variante polimórfica de la $PrP^C$ $K_{222}$ proporciona un efecto dominante negativo sobre la secuencia de $PrP^C$ *wt* que resulta en una reducción de la eficacia de propagación de la tembladera clásica.

Los ratones $K_{176}$-Tg570 (2X) fueron resistentes a todos los aislados de tembladera clásica y EEB inoculados pero completamente susceptibles a la infección con el aislado de tembladera atípica. Estos resultados demuestran que la sustitución aminoacídica asparragina-lisina en el codón 176 ($N/K_{176}$) de la secuencia de $PrP^C$ de cabra confiere un alto nivel de protección frente a la infección con los agentes de tembladera clásica y EEB pero parece aumentar la susceptibilidad a la tembladera atípica.

Los ratones $Q_{211}$-Tg580 (1X) y $R_{171}$-Tg552 (1X) fueron resistentes a la inoculación con una variedad de aislados de tembladera clásica pero susceptibles a la infección con el agente de tembladera atípica lo que demuestra que los cambios aminoacídicos arginina-glutamina en el codón 211 ($R/Q_{211}$) y glutamina-arginina en el codón 171 ($Q/R_{171}$) afectan a la replicación de la $PrP^c$ con los agentes de tembladera clásica pero no con el agente de la tembladera atípica.

A pesar de los bajos niveles de expresión (0.25X) de la $PrP^C$ de la línea $H_{154}$-Tg563, estos ratones fueron susceptibles a algunas cepas de tembladera y de EEB, lo que indica el bajo o nulo efecto de esta variante polimórfica en la infección con priones. Sin embargo, sería necesario generar nuevas líneas de

ratón Tg que expresen la $PrP^C$ mutada $H_{154}$-$PrP^C$ a niveles fisiológicos de cabra para concluir sobre su papel en la infección con priones.

Por último, los ratones $M_{142}$-Tg541 (1X) fueron susceptibles a todos los aislados de tembladera clásica y EEB inoculados aunque mostraron alargamientos en los TS estadísticamente significativos con respecto a los ratones Wt-Tg501 cuando se desafiaron con los aislados de tembladera clásica. Estos retrasos en los TS variaron entre los aislados de tembladera lo que sugiere que el efecto de la variante $M_{142}$-$PrP^C$ en la replicación de los agentes de la tembladera clásica es dependiente de la cepa. Remarcablemente, ninguno de los ratones $M_{142}$-Tg541 sucumbió a la inoculación con el aislado de tembladera atípica lo que indica que el cambio de aminoácido isoleucina-metionina en el codón 142 ($I/M_{142}$) no tiene ningún efecto importante en la replicación de la tembladera y el EEB, pero sí juega un papel determinante en la replicación de la tembladera atípica.

Las inoculaciones en cabras con el caso natural de tembladera clásica mostraron que los animales que portaban la mutación $I/M_{142}$ fueron totalmente susceptibles a la infección con este agente tanto por la vía oral como IC aunque con periodos de incubación más largos que las cabras WT. Estos resultados confirman la conclusión de que la variante $M_{142}$-$PrP^C$ prolonga el tiempo de supervivencia de los animales pero no confiere resistencia. Las cabras portadoras de los genotipos $H_{154}$, $Q_{211}$ y alelos $K_{222}$ fueron resistentes a la tembladera clásica tras la exposición oral. Sin embargo, en comparación con los animales WT, las cabras $H_{154}$ y $Q_{211}$ sólo aumentaron moderadamente sus períodos de incubación tras el desafío IC. Notablemente, sólo unas pocas cabras portadoras de la variante $K_{222}$ desarrollaron la enfermedad con períodos de incubación que eranc uatro a cinco veces más largos que en los animales WT. Estos resultados concuerdan con la resistencia atribuida a las variantes de la $PrP^C$ $Q_{211}$ y $K_{222}$ en los estudios de transmisión en los ratones Tg y refuerzan la buena resistencia de la variante $K_{222}$ a la infección con el agente de tembladera clásica.

Las inoculaciones orales de las cabras WT, $R/Q_{211}$ y $Q/K_{222}$ con el aislado de EEB de cabra mostró que las cabras $R/Q_{211}$ desarrollaron signos clínicos similares a la cabra WT pero con tiempos de incubación más largos. La $PrP^{Sc}$ se observó sólo en los cerebros de estos animales, mientras que sí se detectó infectividad en los tejidos periféricos mediante bioensayo en ratón. Por el contrario, ninguna de las cabras $Q/K_{222}$ mostró ninguna evidencia clínica de enfermedad priónica hasta el final del estudio (86 meses después de la inoculación). Aunque no se observó acumulación de $PrP^{Sc}$ en sus cerebros o tejidos periféricos, sí se determinó una infectividad muy baja en algunos tejidos después de muy largos tiempos post-inoculación (44-45 meses). Estos resultados contrastan con la alta susceptibilidad de los ratones $K_{222}$-Tg516 a la infección IC con EEB de cabra y apoyan la opinión de que otros factores aparte de la secuencia de la $PrP^{C}$ (por ejemplo, la vía de inoculación) influyen en la eficiencia de transmisión de priones. El efecto protector proporcionado por la variante $K_{222}$-$PrP^{C}$ contra el EEB y la tembladera clásica observado en las cabras junto con el efecto protector contra la tembladera clásica obtenido en los ratones Tg prueba la capacidad de esta variante para controlar la transmisión horizontal de ambas enfermedades en rebaños de cabras a través de programas de cría selectiva.

# II. SUMMARY

1. Introduction

Prion diseases or Transmissible Spongiform Encephalopaties (TSEs) are progressive and fatal neurodegenerative diseases that affect humans and other mammal species. These diseases include Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) in humans, as well as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in deer and elk.

A fundamental event in the pathogenesis of prion diseases is the misfolding of host cellular prion protein ($PrP^{C}$) into a pathogenic prion protein ($PrP^{Sc}$) that is able to propagate by recruiting $PrP^{C}$. It is well known that certain amino acid changes in the $PrP^{C}$ sequence can affect the efficiency of $PrP^{C}$ conversion and prion transmission. Indeed, depending on their PrP genotype, individuals from a single population of a species can exhibit different levels of susceptibility to a prion agent, which has important implications for the design of strategies aimed at controlling and eradicating prion diseases via the promotion of PrP genotypes associated with resistance to prion infection. Thus, selective breeding programs have been successfully implemented in sheep herds that have promoted the $A_{136}R_{154}R_{171}$ genotype given its resistance to classical scrapie. As a result, classical scrapie prevalences have declined in many European countries, thereby prompting greater use of these strategies to control other prion diseases such as CWD in cervids or scrapie in goats.

Aside from the PrP amino acid differences between host $PrP^{C}$ and donor $PrP^{Sc}$, prion strains also modulate prion conversion. For example, $A_{136}R_{154}R_{171}$ sheep have greater susceptibility to atypical scrapie than PrP genotypes such as VRQ that are susceptible to classical scrapie. This fact casts a doubt on the achievements of selective breeding programs whose aim is to prevent the infection of sheep with classical scrapie. It seems that prion occurrence would be modulated by a complex combination of the conformational properties of

$PrP^{Sc}$ aggregates and local structures of $PrP^{C}$. It is thus necessary to study PrP sequence changes in combination to prion strains so as to advance our knowledge of the molecular mechanisms governing the susceptibility/resistance to prion infection, which will - if successful - have fundamental implications in control and eradication of prion diseases.

2. Objectives and experimental design

The main objective of this thesis is to improve our understanding of the molecular mechanisms driving the susceptibility/resistance to prion infection. To address this task, a number of specific aims were pursued:

1. To study the individual role of the polymorphic variants found naturally in goat and sheep prion protein gene (*prnp*) in the susceptibility/resistance to scrapie and BSE infection in transgenic mice. For this purpose, we generated a panel of transgenic (Tg) mouse lines expressing either the goat wild type $PrP^{C}$ or $PrP^{C}$ variants containing single amino acid exchanges ($I/M_{142}$, $R/H_{154}$, $R/Q_{211}$, $Q/K_{222}$, $Q/R_{171}$ and $N/K_{176}$); and challenged them with several prion strains. Their susceptibilities were assessed and compared in order to determine the individual role of each $PrP^{C}$ variant in the resistance/susceptibility to prion infection.
2. To confirm in the natural host (goats) the reliability of the results of the mouse models. Experimental inoculations with goats harboring different PrP genotypes with either one scrapie field case or one goat-BSE isolate were performed and their results compared with those obtained from Tg mice.

3. Materials and Methods

Tg mouse lines expressing different $PrP^{C}$ polymorphic variants of goat and sheep ($M_{142}$, $H_{154}$, $R_{171}$, $K_{176}$, $Q_{211}$ and $K_{222}$) were generated by directed mutation of the pMo-GoPrP.Xho plasmid used for obtaining the goat Wt-Tg501 mouse line which expresses the goat wt $PrP^{C}$. Founders were characterized by i) a PCR used to study their capacity to transmit the mutated transgene to the progeny;

ii) Western blot (WB) analysis to measure their $PrP^{C}$ level of expression in the brain; and iii) their behavior and health status, which were monitored for the development of abnormalities. Selected Tg mouse lines were then intracerebrally (IC) inoculated in the right parietal lobe with a variety of prion isolates (consisting of prion infected brain homogenate) that differed in the strain (classical or atypical scrapie, or classical or atypical BSE), geographical origin, $PrP^{Sc}$ genotype, WB $PrP^{res}$ profile, or behavior upon passage in BoPrP-Tg110 mice. A comparative study of the transmission features (survival times, attack rates, $PrP^{res}$ Western-Blot glycoprofile, and histoptahological alterations) was carried out to assess the differences between the Tg mouse lines.

In parallel, we worked in collaboration with ENV (France) and FLI (Germany) to carry out prion transmission studies in goats. On one hand, goats harboring different *prnp* genotypes (WT or *prnp* variants $M_{142}$, $H_{154}$, $Q_{211}$ or $K_{222}$) were challenged with a natural scrapie isolate by the IC or oral routes. For oral challenges, goat kids received the inoculum (scrapie-infected brain) by natural suckling within 48 hours of birth and a second dose at the age of 30 days. For IC challenges, inoculum (the same homogenized scrapie-infected brain) was injected into the temporal cortex of six-month-old goats. Both groups of animals were monitored for the development of neurological diseases and euthanized at the clinical stage of the disease. The presence of $PrP^{Sc}$ was assessed in brains by WB and immunohistochemistry (IHC) analysis.

In parallel, goats harboring WT, $R/Q_{211}$ or $Q/K_{222}$ *prnp* genotypes were orally inoculated with 5ml of pooled goat brain homogenate infected with goat-BSE isolate by natural suckling to assess their relative susceptibility to BSE infection. Goats were culled at different time points during the incubation period and after the onset of clinical signs, and their brains - as well as several peripheral tissues - were analyzed for i) the accumulation of pathological prion protein ($PrP^{Sc}$) using WB and histopathological analysis; and ii) prion infectivity using mouse bioassay in BoPrP-Tg110 mice and non-parametric Mann-Whitney-U test.

4. Results and discussion

All selected Tg mouse lines expressed similar levels in their brains of the same $PrP^C$ sequence except for a single amino acid exchange corresponding to the tested $PrP^C$ polymorphic variant ($I/M_{142}$, $R/H_{154}$, $Q/R_{171}$, $N/K_{176}$, $R/Q_{211}$ and $Q/K_{222}$). None showed any abnormality in their physiological behavior or any histopathological alterations in their brains which rules out the development of a spontaneous neurological disease as a result of the expression of the mutated $PrP^C$.

Wt-Tg501 mice, expressing 1-fold the $PrP^C$ in the goat brain (1X), were susceptible to all tested scrapie and BSE isolates regardless of the aforementioned properties of the isolates. Wt-Tg501 mice were able to replicate all these prion agents and maintain their biological and biochemical properties. As well, this mouse line permitted us to identify at least four classical scrapie strains among the panel of prion isolates. Conjointly, these transmission studies indicate that the Wt-Tg501 mouse line is a highly valuable model for studying the susceptibility of goats and sheep to different scrapie and BSE strains, and also for identifying and characterizing prion strains affecting sheep and goat.

In contrast, $K_{222}$-Tg516 mice, expressing 1X the $K_{222}$-$PrP^C$, were resistant to the primary transmission of all the classical scrapie isolates, classical Cattle-BSE and atypical BSE-L isolates inoculated but not against atypical scrapie agent. Therefore, the single glutamine-to-lysine amino acid substitution at codon 222 ($Q/K_{222}$) provides resistance against classical scrapie, classical cattle BSE and cattle BSE L but not against atypical scrapie or BSE adapted to sheep or goat. Furthermore, heterozygous $Q/K_{222}$ mice were resistant to the inoculation with several scrapie isolates and when succumbed to the prion disease, they did it with lower attack rates (AR) and/or longer survival times (ST) than Wt-Tg501 mice; thus indicating that the $K_{222}$-$PrP^C$ polymorphic variant provides a dominant negative effect over the wild-type $PrP^C$ sequence which results in a poorer efficacy of classical scrapie prion propagation.

None of the $K_{176}$-Tg570 (2X) mice succumbed to any of the classical scrapie or BSE isolates inoculated but were fully susceptible to the infection with atypical scrapie, which demonstrates that the asparagine-to-lysine amino acid substitution at codon 176 ($N/K_{176}$) in the goat $PrP^C$ sequence triggers a high level of resistance to infection with classical scrapie and BSE; although it seems to increase the level of susceptibility to atypical scrapie.

$Q_{211}$-Tg580 (1X) and $R_{171}$-Tg552 (1X) mice were resistant to the inoculation with a variety of classical scrapie isolates but succumbed to the infection with the atypical scrapie isolate. Hence, single arginine-to-glutamine amino acid substitutions at codon 211 ($R/Q_{211}$) and glutamine-to-arginine at codon 171 ($Q/R_{171}$) in the goat $PrP^C$ sequence confer resistance to infection with classical scrapie but not with atypical scrapie.

Despite the low levels of $PrP^C$ expression in their brains (0.25X), $H_{154}$-Tg563 mice were susceptible to some scrapie and BSE isolates, thereby indicating the low or null effect of this polymorphic variant on prion infection. The generation of new Tg mouse lines expressing the $H_{154}$-$PrP^C$ at goat physiological levels is required for concluding on their role in prion infection.

Finally, $M_{142}$-Tg541 (1X) mice were susceptible to all the classical scrapie and BSE isolates inoculated and had statistically significant longer ST compared to Wt-Tg501 mice when scrapie challenged. These delays in ST in animals varied among the scrapie challenges suggesting a strain-dependent effect for $M_{142}$-$PrP^C$ in the replication of classical scrapie agents. More interestingly, none of the $M_{142}$-Tg541 mice succumbed to the inoculation with the atypical scrapie isolate which indicates that the single isoleucine-to-methionine amino acid exchange at codon 142 ($I/M_{142}$) has no major effect on the replication of scrapie and BSE agents but does play a determinant role in the replication of the atypical scrapie agent.

Goat bioasssay with the natural scrapie case showed that the $I/M_{142}$ goats were fully susceptible to the scrapie inoculation by both the IC and oral routes despite having longer incubation periods than WT goats. These results confirm

the finding that the $M_{142}$-$PrP^C$ variant's function is to prolong survival times. Goats carrying the $H_{154}$, $Q_{211}$ and $K_{222}$ *PRP* alleles were all resistant to scrapie infection following oral exposure. However, in comparison to WT animals, the $H_{154}$ and the $Q_{211}$ allele's carriers only increased moderately during the incubation period following the IC challenge. Notably, only very few IC-challenged $K_{222}$ goats developed the disease with incubation periods that were 4-5 times longer than in WT animals. These results agree with the resistance attributed to $Q_{211}$ and $K_{222}$-$PrP^C$ variants in transmission studies in Tg mice and further support the of the good resistance of the $K_{222}$-$PrP^C$ variant to scrapie infection.

Oral inoculations of WT, $R/Q_{211}$ or $Q/K_{222}$ goats with a Goat-BSE isolate showed that $R/Q_{211}$ goats had similar but delayed clinical signs compared to WT goats. $PrP^{Sc}$ was detected only in brains, whereas infectivity was also present in peripheral tissues in both WT and $R/Q_{211}$ goats. By contrast, none of the $Q/K_{222}$ goats showed any evidence of clinical prion disease by the end of the study (86 months post-inoculation). Although no $PrP^{Sc}$ accumulation was observed in their brains or peripheral tissues, very low infectivity was detected in some tissues after very long post-inoculation times (44–45 months). These results contrast the high susceptibility of $K_{222}$-Tg516 mice to the IC infection with Goat-BSE and support the view that factors other than the $PrP^C$ sequence (e.g. the inoculation route) influence prion transmission efficiency. The protective effect provided by the $K_{222}$-$PrP^C$ variant against goat-BSE in goats complements its protective effect against scrapie seen in our studies in goats and Tg mice and is further evidence of the ability of the $K_{222}$-$PrP^C$ variant to control the horizontal transmission of both classical scrapie and goat-BSE diseases in goat herds via selective breeding programs.

# III. INTRODUCTION

## 1. Prion diseases: general aspects

Prion diseases or Transmissible Spongiform Encephalopaties (TSEs) are fatal neurodegenerative diseases that affect numerous mammal species and include Creutzfeldt-Jacob disease (CJD), kuru, Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) in humans, as well as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in deer and elk (Table 1).

TSEs are characterized by long incubation times (from months to decades) and the development of neuropathological alterations consisting of astrogliosis, spongiosis, and neuronal death. Symptoms are primarily neurological and include behavior abnormalities, motor dysfunction, cognitive impairment, and cerebral ataxia. Prion diseases do not produce any immune response and no effective therapy is yet available to treat them.

TSEs are caused by the conversion of the physiological cellular prion protein ($PrP^{C}$) into a pathogenic misfolded isoform (designated $PrP^{Sc}$) that is able to propagate by recruiting more $PrP^{C}$. This conformational change confers $PrP^{Sc}$ with a greater tendency to aggregate, greater insolubility in non-ionic detergents, high resistance to heat and chemical sterilization, and partial resistance to protease digestion. (Pruden et al., 2004)

This revolutionary concept of infectious proteins was firstly described in the Protein-Only Hypothesis proposed by Prusiner and Griffin (Prusiner, 1991). Before this description, prion diseases were thought to be caused by "slow viruses" (Gajdusek and Gibbs, 1968; Kimberlin, 1982). However, given the evidence that the agent causing scrapie was resistant to heat and formaldehyde, as well as to ionizing radiations and UV light (Alper et al., 1967), it was suggested that this agent was neither a virus nor any other infectious agent harboring nucleic acids.

On this basis, Griffith proposed that the scrapie agent could be a protein that self-replicates through autocatalytic conformational changes (Griffith, 1967). To

test this idea, Stanley Prusiner subjected some hamster-adapted scrapie agents to physicochemical inactivation (normally used to destroy proteins) and observed that they lost their infectivity (Prusiner et al., 1981). This way, scrapie became to be considered an infectious protein and a new term **"PRION"** was coined to define this atypical agent.

Although CJD and kuru were first described in 1920 and 1957, respectively, TSEs did not become a pressing concern in public health until the development of the BSE epidemics in the 1980s in the United Kingdom. The magnitude of this disease, which rapidly spread among cattle in numerous European countries as well as the USA, Canada and Japan, was even higher when transmitted to humans, thereby giving rise to a new variant of Creutzfeldt-Jacob disease (vCJD).

Since then, the group of TSEs identified has continued to grow. Indeed, two atypical variants of BSE have recently been reported in cattle (L-BSE (Casalone et al., 2004) and H-BSE (Biacabe et al., 2004)). Thus, nowadays more than 17 TSEs have been reported to affect an increasing number of wild and domestic animal species, as well as humans (Table 1).

Table 1. Etiology of prion diseases

| Disease | Host | Etiology | Year of description |
|---|---|---|---|
| **Bovine Spongiform Encephalopathy (BSE)** | Cattle | Infection with prions of unknown origin | 1986 |
| **Bovine Amyloidotic Spongiform Encephalopathy (BSE-L)** | Cattle | Infection with prions of unknown origin | 2004 |
| **Atypical Bovine Spongiform Encephalopathy Type-H (BSE-H)** | Cattle | Infection with prions of unknown origin | 2004 |
| **Scrapie** | Sheep, goats, mouflons | Infection with prions of unknown origin | 1972 |
| **Atypical scrapie** | Sheep and goats | Spontaneous $PrP^{C}$ to $PrP^{Sc}$ conversion or somatic mutation in *prnp*[a] | |
| **Chronic Wasting Disease (CWD)** | Cervids | Infection with Prions of unknown origin | 1967 |
| **Transmissible Mink Encephalopathy (TME)** | Minks | BSE or scrapie-contaminated meat and bone meal | 1947 |
| **Feline Spongiform Encephalopathy (FSE)** | Cats, cheetahs, ocelots, pumas and tigers | BSE-contaminated meat and bone meal | 1990 |
| **Exotic ungulate Spongiform Encephalopathy (EUE)** | Kudu, eland, gemsbok, nyala oryx, bison | BSE-contaminated meat and bone meal | 1986 |
| **TSEs in non-human primates (NHP)** | Lemurs and Rhesus macaques | BSE-contaminated meat and bone meal | 1996 |
| **Kuru** | Human | Ritualistic cannibalism | 1900s |
| **Variant CJD (vCJD)** | Human | BSE-contaminated meat and bone meal | 1996 |
| **Sporadic CJD (sCJD)** | Human | Spontaneous $PrP^{C}$ to $PrP^{Sc}$ conversion or somatic mutation in *prnp* | 1920 |
| **Familial CJD (fCJD)** | Human | Mutations in *prnp* | 1924 |
| **Iatrogenic CJD (iCJD)** | Human | Infection with prions of human origin through medical practices (surgeries, transplants) | 1974 |
| **Gerstmann-Sträussler-Scheinker syndrome, (GSS)** | Human | Mutations in *prnp* | 1936 |
| **Fatal familial insomnia (FFI)** | Human | Mutations in *prnp* | 1986 |
| **Sporadic Fatal Insomnia (sFI)** | Human | Spontaneous $PrP^{C}$ to $PrP^{Sc}$ conversion or somatic mutation in *prnp* | 1999 |
| **Variably protease-sensitive prionopathy (VPSPr)** | Human | Spontaneous $PrP^{C}$ to $PrP^{Sc}$ conversion or somatic mutation in *prnp* | 2008 |

[a] *prnp*:prion protein gene

## 2. Prion conversion: from $PrP^{C}$ to $PrP^{Sc}$

According to the Prion Protein-Only Hypothesis, prion diseases are uniquely caused by the post-translational conversion of the cellular prion protein ($PrP^{C}$) into a pathogenic misfolded isoform ($PrP^{Sc}$) that has the ability to self-propagate (Prusiner, 1991).

To date, several studies have supported the Prion Protein-Only Hypothesis, including the successful induction of neurodegenerative diseases only from recombinant amyloid forms of prions (Castilla et al., 2005b; Colby et al., 2009; Legname et al., 2004) or in combination with certain lipids and RNA factors (Wang et al., 2010). Nevertheless, some findings suggest that the misfolded $PrP^{res}$ protein alone is not necessarily infectious and needs some cofactors to be able to self-propagate (Deleault et al., 2012; Saa et al., 2012; Telling et al., 1995); in this case, $PrP^{res}$ formation and infectious agent replication are two different processes. The existence of high infectivity when no or low amounts of $PrP^{res}$ are detected supports this view. On this basis, some authors have proposed that infectivity in prion diseases is caused by other non-prion structures such as the viruses of the vast metagenome (reviewed in reference (Manuelidis, 2013)).

Despite these arguments, the conversion of $PrP^{C}$ into $PrP^{Sc}$ is essential for prion disease development and prion diseases are entirely dependent on the expression of endogenous $PrP^{C}$, as confirmed by the total resistance to TSEs of *prnp* knock-out mice (Bueler et al., 1993; Prusiner et al., 1993).

## 2.1. Cellular prion protein ($PrP^C$)

### *2.1.1. Prion protein gene (prnp)*

The prion gene family consists of three members: *prnp* gene that encodes the cellular prion protein ($PrP^C$), *prnd* gene that encodes the Doppel protein, and *sprn* gene that encodes the Shadoo protein (Fig. 1).

*Prnp* gene has been well conserved throughout evolution in mammals and paralog genes have been described in birds (Gabriel et al., 1992), amphibians (Strumbo et al., 2001), reptiles (Simonic et al., 2000), and fish (Cotto et al., 2005). *Prnp* is located on chromosome 2 in mice, 20 in humans, and 13 in ruminants (Sparkes et al., 1986). In mammalian species, *prnp* gene contains two (humans) or three (mice) exons; nevertheless, the complete $PrP^C$ protein is encoded by the terminal exon where the entire open reading frame (ORF) is located in all cases. The other exons harbor untranslated sequences such as promoter and termination sites. At the promoter level, *prnp* is characterized by the absence of a TATA box (Basler et al., 1986), which is substituted by a GC-rich repeats regulator sequence with potential binding sites for many transcription factors such as Sp1, AP1, and AP2 (Inoue et al., 1997; Mahal et al., 2001; McKnight and Tjian, 1986; Saeki et al., 1996; Westaway et al., 1994).

*Prnd* gene lies 16 kb downstream of the murine *prnp* locus (Moore et al., 1999) and encodes a 179-residue PrP-like protein designated Doppel. *Prnd* postembryonic expression is nil in central nervous system (CNS) and principally confined to the testis, where Doppel seems to play a determinant role in spermatogenesis (Behrens et al., 2002). Doppel exhibits less than 25% homology with the $PrP^C$; however, its upregulation in the CNS in the absence of the $PrP^C$ produces late-onset ataxia in transgenic mice, suggesting that this protein may be associated to neurodegeneration (Moore et al., 1999).

*Sprn* gene is located on chromosome 7 in mice, 10 in humans, and 26 in ruminants. It encodes a protein of 130–150 amino acids named Shadoo, which harbors very similar domains to the $PrP^C$ protein (Fig. 1). While Doppel

resembles the α-helical C-terminal half of $PrP^C$, Shadoo is reminiscent of the flexible N-terminal half. *Sprn* gene has partial homology to the $PrP^C$ hydrophobic domain, a series of N-terminal repeats, a C-terminal N-linked glycosylation, and a GPI anchor. Moreover, Shadoo is extracellular and GPI-anchored and its pattern of expression in CNS seems to overlap that of $PrP^C$ (Watts et al., 2007; Young et al., 2009). These similarities may explain the functional analogy between Shadoo and $PrP^C$ (Passet et al., 2012; Young et al., 2009).

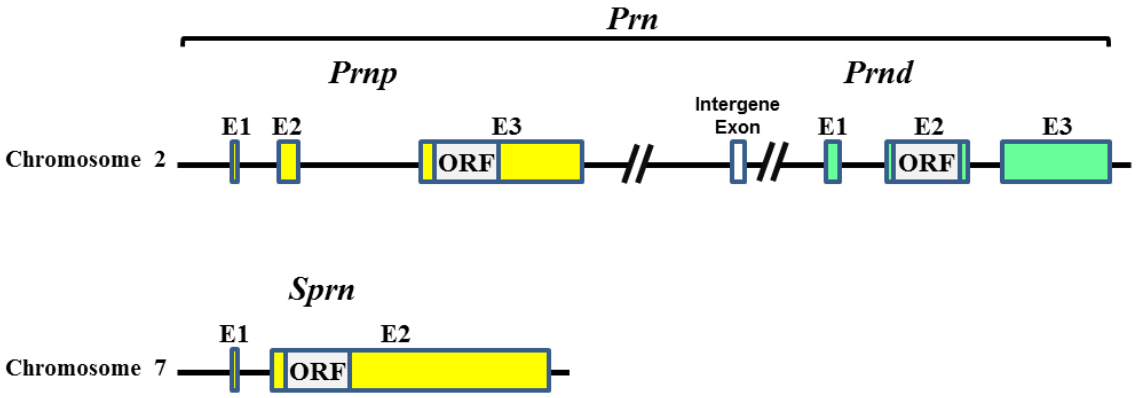


**Figure 1. Schematic representation of mouse *prnp*, *prnd*, and *sprn* genes.** The *prn* locus is located on chromosome 2 and contains *prnp* and *prnd* genes that encode $PrP^C$ and Doppel proteins, respectively. *Sprn* is located on chromosome 7 and encodes the Shadoo protein. E: Exon (adapted from (Watts et al., 2007)).

### *2.1.2. $PrP^C$ structure and biosynthesis*

*Prnp* transduction takes place in the endoplasmic reticulum (ER) where a 235–264-amino-acid $PrP^C$ protein containing two glycosylation sites and a GPI anchor is produced (Colby and Prusiner, 2011). $PrP^C$ is post-translationally modified in the ER, a process that includes the cleavage of a 22-aa signal peptide, the removals of the 22-aa N-terminal signal peptide and a C-terminal hydrophobic segment, the formation of a single disulfide bond, glycosylation at one or two sites, and the attachment of a glycosyl phosphatidyl inositol (GPI) anchor (Stahl et al., 1987) (Fig. 2.A).

After these post-translational modifications, the mature prion protein has ≈ 210 amino acids and a molecular weight of around 26–37 kilodaltons (K) depending on its glycosylation state (un-, mono- or diglycosilated). Then, mature $PrP^C$ traffics through the Golgi apparatus to the cell surface where it is inserted and targeted in the detergent-resistant cholesterol-rich lipid domains (rafts) of the plasma membrane through its GPI anchor (Fig. 2B).

Structural and biochemical studies in recombinant PrP or extracts have shown that the $PrP^C$ protein has two distinguishable parts: i) a long unstructured flexible N-terminal tail of ≈100 residues with the copper-binding octapeptide repeats (PHGGGWGQ), and ii) a globular domain of ≈100 residues that harbors the hydrophobic region and the short flexible C-terminal domain composed of three α-helices and two antiparallel β-sheets separated by short loops (Riek et al., 1998) with a single disulfide bond linking cysteine residues from α-helices 2 and 3 (Fig. 2.A).

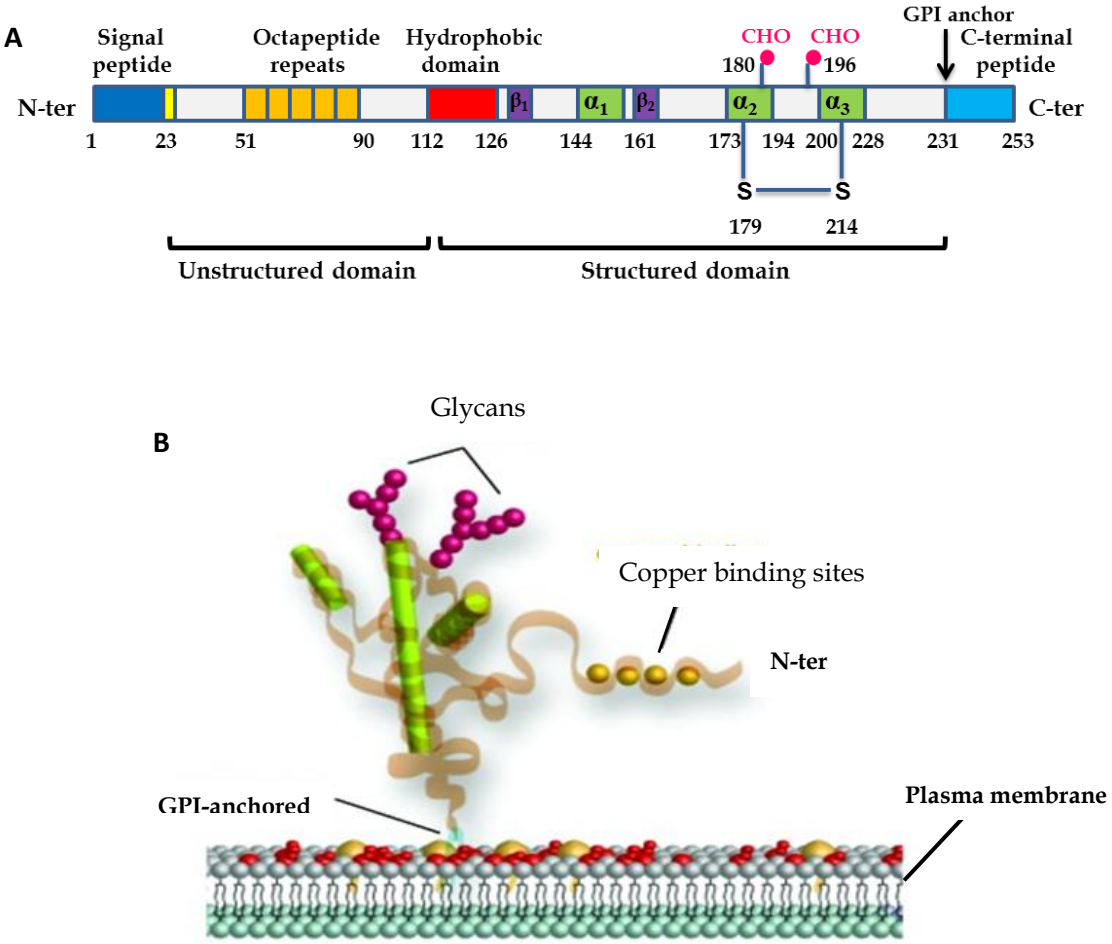


**Figure 2. Mouse PrP$^C$ structure. A.** Schematic representation of mouse PrP$^C$ primary structure. The PrP$^C$ structure consists of 1) an unstructured N-terminal tail encompassing a polybasic region (residues 23–27, yellow) and a tandem of five repeats of copper-binding octapeptide sequences (residues 51–90, orange) and a positively charged region (residues 90–112, white); and 2) a structured C-terminal domain that spans a highly conserved hydrophobic domain (residues 112–126, red); three α-helices ($\alpha_1$, $\alpha_2$ and $\alpha_3$; residues 144, 173 and 200, green), two short β-strands ($\beta_1$ and $\beta_2$; residues 127 and 161, purple) and a disulfide bond linking cysteine residues from α-helices 2 and 3 (S-S, residues 179 and 214). Signal peptide (residues 1–22, cyan) and a C-terminal peptide (residues 231–253, light blue) are removed during biosynthesis, followed by attachment of a glycosylphosphatidylinositol (GPI) moiety. PrP$^C$ also contains two N-linked oligosaccharide chains (at Asn180 and Asn196, pink) and a disulfide bond between residues 178 and 231 in mouse numbering. Adapted from (Petit et al., 2013). B. PrP$^C$ anchored in lipid raft of cellular membrane (three-dimensional structure) extracted from (Petit et al., 2013).

### *2.1.3. $PrP^C$ trafficking and metabolism*

$PrP^C$ half-life extends to one or two hours in a primary culture of neurons or splenocytes (Parizek et al., 2001) and five to six hours in neuroblastoma cells N2a (Borchelt et al., 1990). After its cleavage in the plasma membrane, $PrP^C$ is believed not to remain on the cell surface but cycles between the plasma membrane and an endocytic compartment (Fig. 3) (Shyng et al., 1993).

$PrP^C$ is internalized into an endocytic compartment from which most of the molecules are recycled intact to the cell surface; a small percentage of the endocytosed molecules are proteolytically cleaved via late endosomes and lysosomes (Fig. 3). $PrP^C$ endocytosis lasts three to five min (Sunyach et al., 2003) and, depending on the cell type, may occur through clathrin-dependent pathways (Marella et al., 2002; Peters et al., 2003) or be mediated by "caveolae-like" domains (Shyng et al., 1993) (Fig. 3).

Cellular processing of $PrP^C$ involves two well-documented proteolytic cleavage events. α-cleavage occurs at residues $His_{114}$ and $Val_{115}$ for the ovine $PrP^C$ (Tveit et al., 2005) and produces a di- (25–27 K), mono- (21–23 K), or un-glycosylated (17 K) C-terminal fragment (C1) (Chen et al., 1995; Kuczius et al., 2007; Laffont-Proust et al., 2005) and a 9 K N-terminal fragment (N1), which is mostly released from the cell by shedding (Vincent et al., 2000). On the other hand, β-cleavage occurs within or adjacent to the octapeptide repeats (at $Gly_{92}$ of the ovine $PrP^C$) and generates a 21-K C-terminal fragment (C2) and the corresponding 7 K N-terminal fragment (N2) (Chen et al., 1995). This type of cleavage is less common in healthy animals than α-cleavage and appears to occur as a response to oxidative stress (Watt and Hooper, 2005), while α-cleavage may be mediated by the ADAM family (Vincent et al., 2001).

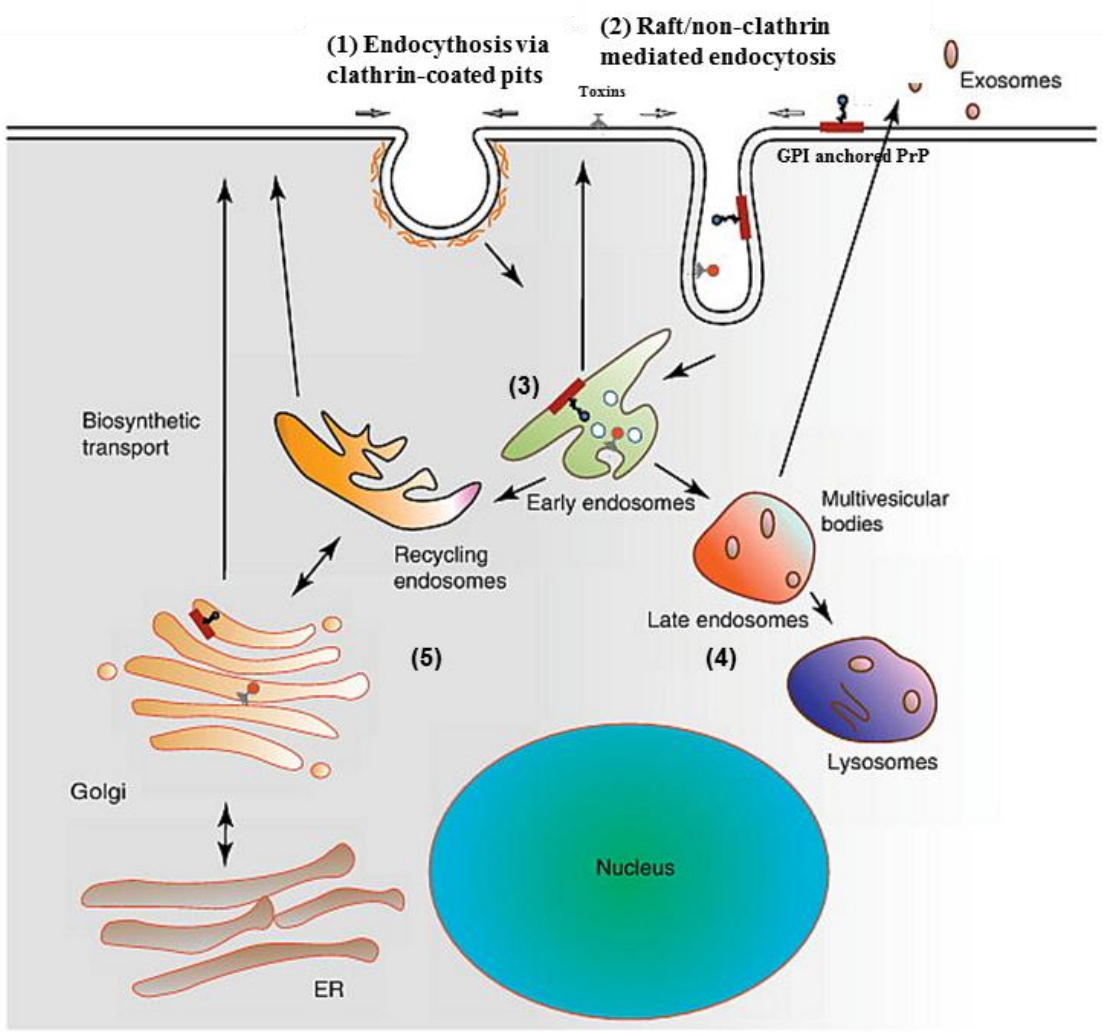


**Figure 3. PrP$^C$ trafficking in the cell.** PrP$^C$ can be internalized into early endosomes via clathrin-coated pits (1) or via non-clathrin-coated pits (2) from which PrP$^C$ can be routed either for degradation (3) *via* late endosomes and lysosomes or for recycling (4) *via* recycling endosomes and Golgi apparatus (5). Adapted from (Rajendran et al., 2012).

.

### *2.1.4. $PrP^C$ functions*

$PrP^C$ has a high degree of conservation in mammalian species, which suggests that it has some important function maintained through evolution. $PrP^C$ is mostly expressed in the CNS, i.e. in the neurons and glia (Brown et al., 1990; Kretzschmar et al., 1986), but also in the lymphoreticular system (LRS), skeletal muscle, heart, kidney, digestive tract, skin, blood plasma, mammary gland, and endothelia (Nuvolone et al., 2009). Despite its ubiquitous expression and distribution, its physiological function is not yet clear.

In an attempt to answer this question, several $PrP^C$ knockout (KO) mice lines were generated. In general $PrP^C$ KO mice are viable, fertile, and have no apparent gross developmental or anatomical abnormalities; the exception is one Tg mouse line that has late onset ataxia associated with a loss of Purkinje cells in the cerebellum, which was finally attributed to the artefactual upregulation of the adjacent Doppel gene (Moore et al., 1999). More recently, goats – which naturally devoid $PrP^C$ – have been documented in Norway (Benestad et al., 2012). These goats showed no apparent abnormal behavior or any other characteristics that differentiated them from their flock-mates.

However, when studied in detail, $PrP^C$ KO mice exhibit dysfunctions in their circadian rhythms, memory, cognition, synaptic transmission, olfaction or immunologic alterations. Moreover, altered levels of nuclear factor NF-kappaB, Mn superoxide dismutase, and COX-IV, Cu/Zn superoxide dismutase activity, p53, and melatonin have also been detected in $PrP^C$ KO mice (Linden et al., 2008; Nicolas et al., 2009; Steele et al., 2007).

Some authors have associated $PrP^C$ with functions related to cell adhesion, enzymatic activity, copper metabolism, proliferation, survival, and differentiation and programmed cell death. In the nervous system, $PrP^C$ seems to actively participate in signal transduction (Collinge et al., 1994), synaptic plasticity (Prestori et al., 2008), neurogenesis, neurite outgrowth (Chen et al., 2003), neuroprotection against pro-apoptotic stimuli, ischemic trauma, and

reactive oxygen species (Chiarini et al., 2002; McLennan et al., 2004), and copper metabolism (Brown et al., 2001).

Outside the nervous system, $PrP^C$ may modulate the response of immune cell precursors to extracellular signaling molecules (Ballerini et al., 2006; Mattei et al., 2004) and also control the hematopoietic cell differentiation, which includes the self-renewal of bone marrow hematopoietic cells (Zhang et al., 2006), thymic differentiation (Jouvin-Marche et al., 2006), and the repression of phagocytosis and inflammatory response (de Almeida et al., 2005).

Due to this ability to modulate cell proliferation and apoptosis, $PrP^C$ has been linked to cancer development and tumor progression, as well as to metastatic processes (Mehrpour and Codogno, 2010; Muras et al., 2009; Pan et al., 2006). Moreover, new findings suggest that $PrP^C$ may also modulate Alzheimer´s disease progression by promoting amyloid-β peptide production from amyloid precursor protein (APP) (Parkin et al., 2007) and by regulating effects of amyloid-β soluble oligomers on synapse alteration (Lauren et al., 2009). Hence, although it is known that $PrP^C$ participates in many fundamental biological processes, its physiological role still needs to be fully elucidated.

## 2.2 Misfolded prion protein ($PrP^{Sc}$)

### *2.2.1. $PrP^{Sc}$ structure*

$PrP^{C}$ conversion into $PrP^{Sc}$ is a post-translational process, that is, both isoforms share an identical amino acid sequence within an individual but differ in their conformation. The structure of physiological $PrP^{C}$ was deduced in 1996 (Riek et al., 1996) (Fig. 4), but the high-resolution three-dimensional structure (3D) of the abnormal $PrP^{Sc}$ remains unknown, along with the mechanistic details of $PrP^{Sc}$ self-propagation.

Deciphering the structural features of $PrP^{Sc}$ is a key issue in understanding the molecular basis of prion formation and propagation. However, the insolubility and the propensity of the $PrP^{Sc}$ isoform to aggregate prevent the use of high-resolution techniques such as NMR or X-ray crystallography. Therefore, only partial structural information is available from low-resolution techniques that in general do not agree on a single feature or type of β-structure to explain the properties of the infectious prion (reviewed in (Requena and Wille, 2014)).

Studies of the secondary structure of $PrP^{Sc}$ suggest that $PrP^{C}$ conversion into the pathological isoform $PrP^{Sc}$ mainly triggers changes in the N-terminal half of the protein such as the folding of a portion of the N-terminal tail from residues 90 to 121 (and possibly part of the first a-helix) into $\beta$-sheet (Peretz et al., 1997). Thereby, unlike $PrP^{C}$, which contains around 47% $\alpha$-helices and 3% $\beta$-sheets (Riek et al., 1996), $PrP^{Sc}$ is mostly folded into $\beta$-sheets, holding 17–30% $\alpha$-helix and 43-54% extended $\beta$-structure (Pan et al., 1993) (Fig. 4).

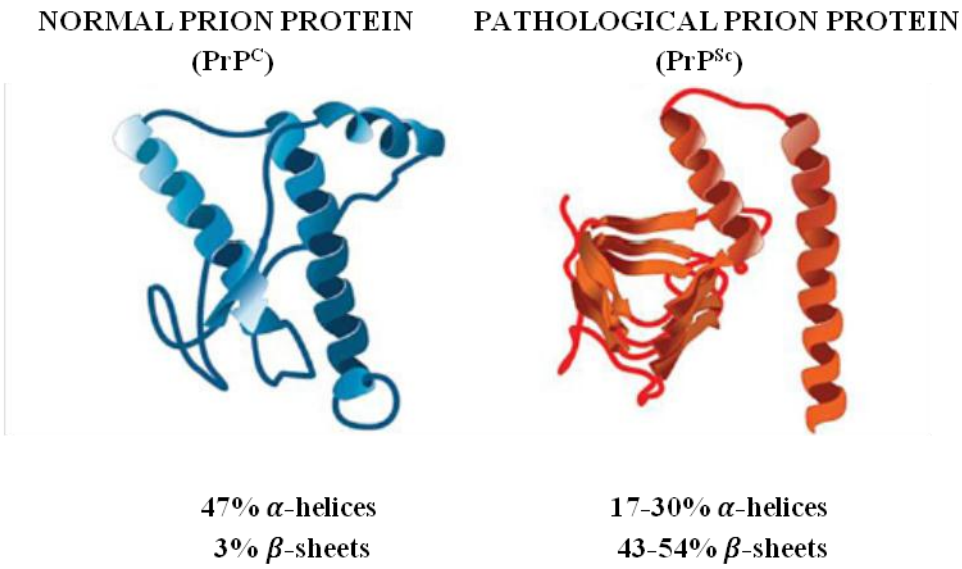


**Figure 4. Proposed structure of PrP$^{C}$ and PrP$^{Sc}$** (adapted from (Prusiner, 2004)).

### *2.2.2. PrP$^{Sc}$ characteristics*

PrP$^{C}$ and PrP$^{Sc}$ differ profoundly in their biochemical and biophysical properties. PrP$^{C}$ is monomeric, proteinase-sensitive, and soluble in non-ionic detergents, whereas conformational misfolding confers PrP$^{Sc}$ with an increased tendency to aggregate, partial resistance to proteinase digestion, insolubility in non-ionic detergents, and high resistance to heat and chemical sterilization (Caughey and Chesebro, 2001; Collinge, 2001; Prusiner, 1998b).

Indeed, PrP$^{Sc}$ digestion with proteinase K (PK) produces a C-terminally non-degraded fragment (PrP$^{27\text{-}30}$ or PrP$^{res}$) beginning around residues 81–89 (in the mouse PrP sequence), which is widely used as a biochemical marker for prion detection and characterization (Gielbert et al., 2009). Intriguingly, several studies have produced evidence of the existence of the involvement of PK sensitive PrP$^{Sc}$ (PrP$^{sen}$) in prion diseases (Gambetti et al., 2003; Pastrana et al., 2006; Safar et al., 2005; Tzaban et al., 2002). PrP$^{sen}$ was first reported by Lasmézas, who observed the presence of clinical disease in the absence of detectable PrP$^{res}$ during the transmission of BSE to mice (Lasmezas et al., 1997. PrPsen has been observed in scrapie-infected sheep {Thackray, 2007 #26292).

$PrP^{sen}$ has also been observed in scrapie-infected sheep and hamsters (Safar et al., 1998). The extension of $PrP^{sen}$ involvement depends on the disease but can represent up to 90% of the total $PrP^{Sc}$ in some sCJD cases (Safar et al., 2005). $PrP^{sen}$ is detected by conformation-dependent immunoassay (CDI), which provides the differential binding sites of prion proteins after denaturation (Safar et al., 1998).

### 2.3. $PrP^{C}$ to $PrP^{Sc}$ conversion site

The exact site at which $PrP^{C}$ interacts with $PrP^{Sc}$ remains unclear. Although analysis of the distribution pattern of $PrP^{C}$ and $PrP^{Sc}$ in cell cultures has contributed to the search for this conversion site, the poor immunoreactivity of aggregated $PrP^{Sc}$ and the difficulties to immunologically distinguish $PrP^{C}$ from $PrP^{Sc}$ have hampered this task.

Most evidence indicates that the interaction between $PrP^{C}$ and exogenous $PrP^{Sc}$ takes place in detergent-resistant rafts, either extracellularly, on the plasma membrane where $PrP^{C}$ is GPI-anchored (Caughey and Raymond, 1991), and/or intracellularly in endosomal vesicles (Borchelt et al., 1992; Caughey et al., 2009; Caughey and Raymond, 1991; Manuelidis et al., 1976; Taraboulos et al., 1992; Vey et al., 1996). These disagreements in proposed cellular models suggest that conversion may well occur along multiple pathways.

## 2.4. Cofactors involved in prion conversion

Some authors have proposed that non-PrP molecules known as cofactors could be involved in the conversion of $PrP^{C}$ into $PrP^{Sc}$. The first evidence of the existence of cofactors arose from prion transmissions in transgenic mice that co-express human and mouse $PrP^{C}$. These mice were resistant to inoculation with human prions; on the other hand, mice expressing only human $PrP^{C}$ developed the prion disease (Telling et al., 1994). These results suggest that mouse $PrP^{C}$ was able to inhibit the human $PrP^{C}$ by binding to another unknown factor. Nevertheless, the relatively low levels of specific infectivity obtained during *in vitro* studies using only pure $PrP^{C}$ molecules as a substrate (Colby et al., 2010; Kim et al., 2010; Makarava et al., 2010) also supports the involvement of cofactors in prion replication.

Several compounds including RNA, homopolymeric nucleic acids such as poly(A) and poly(dT), and nonspecific DNA have shown *in vitro* capacity to promote prion replication (Adler et al., 2003; Cordeiro et al., 2001; Deleault et al., 2005; Deleault et al., 2003). By contrast, other molecules such as synthetic nucleic acids, heparin, albumin, fatty acids, and metal ions may attenuate prion replication *in vitro* (Abid et al., 2010). Although protein chaperones such as Hsp104 and GroEL (Parsell and Lindquist, 1993) are able to promote *in vitro* the conversion process of hamster $PrP^{C}$, chemical chaperones including sucrose, trehalose, and dimethyl sulfoxide inhibit this conversion process (DebBurman et al., 1997). Heparan sulfate proteoglycans (HSPG) have been shown to be able to bring $PrP^{C}$ and $PrP^{Sc}$ together and to act as a scaffold for the $PrP^{Sc}$ fibrillization (Wong et al., 2001). HSPGs are able to bind $PrP^{Sc}$ on the cell surface and facilitate its internalization by macropinocytosis (Horonchik et al., 2005; Wadia et al., 2008) and also to bind $PrP^{C}$, thereby enhancing subcellular trafficking in clathrin-coated pits (Shyng et al., 1994).

The specific identity and functions of cofactors have not yet been fully deciphered. Cofactors could act either as a catalytic molecule that binds both

$PrP^{C}$ and $PrP^{Sc}$ and brings them together, thereby lowering the activation energy for the conversion process, or may just bind $PrP^{Sc}$ and so stabilize its conformation. Cofactors might also play a pivotal role in the strain properties of newly generated $PrP^{Sc}$. The strain properties of recombinant $PrP^{Sc}$ generated from recombinant $PrP^{C}$ by serial Protein Misfolding Cyclic Amplification in the presence of cofactors such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol and RNA molecules were altered in subsequent *in vitro* passages by changing the cofactor element to phosphatidylethanolamine (Saborio et al., 2001).

The question remains whether the cofactor molecule is a component of the infectious particle or whether it is a molecule provided by the host. The latter case would suggest that the infectious agent causing prion diseases consists of a complex $PrP^{Sc}$-cofactor, a hypothesis that contradicts the Protein-Only Hypothesis.

## 2.5. Prion conversion models

Prion diseases may occur as familial (inherited), spontaneous, or acquired (infectious) diseases. The mechanism of conversion from physiological $PrP^{C}$ to pathological $PrP^{Sc}$ has not yet been clarified and two models have been proposed to explain it (Fig. 5):

**- Template-directed refolding model**: this model postulates that the monomeric $PrP^{Sc}$ acts as a conformational template, promoting the conversion of endogenous monomeric $PrP^{C}$ into a conformational intermediate (heterodimer) and finally originating further $PrP^{Sc}$ (Fig. 5). The $PrP^{Sc}$ dimer would then interact with other dimers and thus form larger aggregates. In healthy animals, a high-energy barrier exists that prevents the spontaneous conversion of $PrP^{C}$ into $PrP^{Sc}$. However, disease-linked mutations of the *prnp* could increase the stability of the $PrP^{C}$ mutated forms, either via the interaction between $PrP^{C}$ monomeric components and/or by abrogating the kinetic barrier that inhibits the conversion of $PrP^{C}$ into $PrP^{Sc}$, and so give rise to inherited or spontaneous prion diseases (Bradley and Wilesmith, 1993). In infectious forms of prion diseases, the exogenous $PrP^{Sc}$ has been proposed as the element that leads the misfolding process (Safar, 1996).

**- Seeded nucleation-polymerization model:** this model proposes that $PrP^{C}$ and $PrP^{Sc}$ coexist in a reversible thermodynamic equilibrium that in a physiological state is shifted toward the $PrP^{C}$ conformation limiting the amount of $PrP^{Sc}$. Therefore, according to this model, the monomeric $PrP^{Sc}$ would represent a minor and transient isoform of PrP and would only be destabilized in the event of the organization of several monomeric $PrP^{Sc}$ into highly ordered seeds during the nucleation phase (Fig. 5). These infectious seeds would act as stable nuclei called amyloids that displace the equilibrium to the accumulation of the pathological isoform. The fragmentation of the amyloid aggregates increases the number of replication units, which can recruit further $PrP^{Sc}$ and thus confer the infectious capacity of the prion (Glatzel and Aguzzi, 2001). Thermodynamic

disequilibrium toward $PrP^{Sc}$ would be caused by disease-linked mutations that destabilize $PrP^{C}$, thereby increasing its tendency to misfold (Apetri et al., 2004; Liemann and Glockshuber, 1999; Swietnicki et al., 1998; Zhang et al., 2000). The critical (rate-limiting) nucleation step is responsible for the "lag phase" observed in the spontaneous conversion reaction (Cobb and Surewicz, 2009) that would be shortened or even abolished by "seeding" exogenous preformed $PrP^{Sc}$ aggregates in acquired TSEs (Collinge and Clarke, 2007).

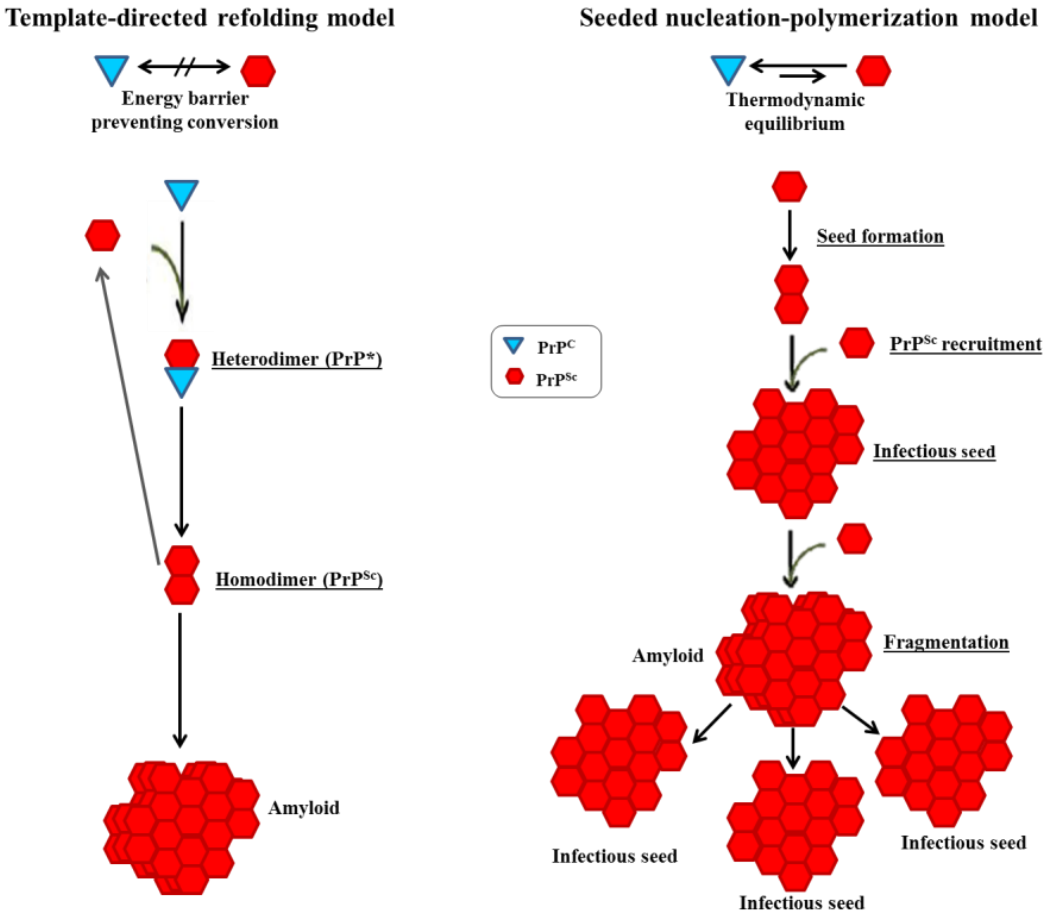


**Figure 5.** Models proposed for the $PrP^{C}$ conformational conversion into $PrP^{Sc}$ (adapted from Aguzzi *et al.*, 2004).

### 3. Pathogenesis of prion diseases

Prion diseases may occur as either inherited or sporadic disorders, or be acquired by infection. In all forms, the key event in the pathogenesis of prion diseases is the conversion of the physiological $PrP^{C}$ into pathological $PrP^{Sc}$. While $PrP^{C}$ is expressed ubiquitously throughout the organism, the main target tissue for $PrP^{Sc}$ replication is the brain.

In acquired TSEs the most likely route of infection by prions is the ingestion of prion-contaminated products such as grass, soil, meat, or bones. Scarification of the skin and infection of mucous membranes/conjunctiva via direct contact with prion-contaminated excretions and secretions (saliva, feces, urine, blood, placenta, and milk) have also been suggested as alternative routes of infection (Mohan et al., 2004; Scott et al., 1993a; Taylor et al., 1996). The spread of prions from the infection site to the brain depends on several factors including entry site, dose, strain, and host genotype.

When orally acquired, infectious prions penetrate the mucosal barrier of the stomach in one of three possible ways: the uptake of M-cells (Neutra et al., 1996), via dendritic cells (not yet demonstrated), or endocytosed in vesicular structures by a ferritin-dependent mechanism (Mishra et al., 2004). Infectious prions reach the gut-associated lymphoid tissues (GALT) of the tonsil and the Peyer's patches in the intestines, where they accumulate and replicate (Andreoletti et al., 2000; Hadlow et al., 1982; van Keulen et al., 2002).

Cell tropism of prion strains seems to play a determinant role at this point. Scrapie is a clear lymphotropic prion agent, i.e. it colonizes lymphoid organs immediately after the peripheral infection and before CNS invasion. By contrast, BSE is neurotropic and invades the brain without any major replication in the LRS. Thus, in naturally infected cattle, replication of the BSE agent in the lymphoid tissues is minimal-to-absent. Only cattle experimentally infected with high doses have BSE infectivity in their ileum or tonsils (Espinosa et al., 2007b; Wells et al., 1994; Wells et al., 1998; Wells et al., 2005).

After replication in the GALT, prions are drained from the GALT-draining retropharyngeal and mesenteric lymph nodes (Andreoletti et al., 2000; van Keulen et al., 2002), from where neuroinvasion occurs through the enteric nervous system (ENS) of the gut (Andreoletti et al., 2000; Iwata et al., 2006; Schmerr et al., 1997). After the infection of the ENS, prions ascend along the parasympathetic (neurons of the dorsal motor nucleus of the nervus vagus, DMNV) and sympathetic efferent (neurons of the intermediolateral column neuronal, IMLC) pathways to the brain and to the spinal cord (Hoffmann et al., 2007; van Keulen et al., 2002). From these sites in the CNS, the infection both ascends and descends and eventually affects the entire neuraxis.

$PrP^{Sc}$ spread to peripheral sites may take place before and/or after neuroinvasion. For example, in scrapie-infected sheep and goats, $PrP^{Sc}$ deposition has also been observed in numerous peripheral structures such as nictitating membrane, muscles, placentas (Andreoletti et al., 2002), skin (Garza et al., 2014; Thomzig et al., 2007), mammary glands (Ligios et al., 2005), distal ilium, proximal colon (van Keulen et al., 2008a), pancreas, heart, and urinary bladder (Garza et al., 2014). The peripheral nervous system appears to be the main mechanism of $PrP^{Sc}$ dissemination in hosts; however, inflammation or the presence of prions in the blood could underlie wider dissemination and more pronounced accumulation of $PrP^{Sc}$ deposits in the body (Garza et al., 2014; Ligios et al., 2005). Indeed, prion infectivity has been reported in humans, primates (Bons et al., 2002), sheep (Houston et al., 2000), goats (Dassanayake et al., 2012), rodents (Brown et al., 1998), and cervids (Bannach et al., 2012; Dassanayake et al., 2012; Dassanayake et al., 2011; Jackman et al., 2006; Lacroux et al., 2012; Mathiason et al., 2010; Schmerr et al., 1997).

Notably, the *prnp* genotype of the host modulates the $PrP^{Sc}$ distribution in scrapie-infected sheep. For example, sheep carrying the $V_{136}R_{154}Q_{171}$ or $A_{136}R_{154}R_{171}$ genotypes exhibited minimal or no involvement of the lymphoid tissues in agent replication (van Keulen et al., 1996). Similar distribution modifications were observed in goats harboring the $H_{154}$, $Q_{211}$ or $K_{222}$ *prnp*

mutations intracerebrally inoculated with scrapie (Lacroux et al., 2014a). Hence, both prion strain and host genotype strongly affect the distribution of infectious prions in the organism via a mechanism that is as yet unknown.

**4. Neurotoxicity**

The neurotoxic mechanism of pathological prions and the responsible toxic forms of PrP are not yet well established. $PrP^{Sc}$ deposition appears as the earliest event in the pathological cascade of prion diseases, followed by microglial activation and the appearance of spongiform change or vacuolation. Different pathways including cellular systems associated with synapses, protein processing, oxidative stress, autophagy, and apoptosis (Kovacs and Budka, 2009) seem to be involved in this tissue damage; nevertheless, the neurotoxic mechanism is still not well known.

$PrP^{Sc}$ aggregates are commonly designated as the primary cause of neurodegeneration due to the temporal and anatomical correlation between the accumulation of this pathological form and the development of neuropathological changes. However, in some cases significant pathology and/or clinical dysfunction develop even with little accumulation of $PrP^{Sc}$; in other cases, subclinical infections have abundant $PrP^{Sc}$ but little symptomatology. Moreover, $PrP^{Sc}$ is innocuous in the absence of GPI-linked $PrP^{C}$, suggesting that PrP oligomers and fibrils are not toxic *per se* (and may require $PrP^{C}$ as a mediator of the toxic signal (Brandner et al., 1996)).

On the other hand, growing evidence supports the neurotoxic role of multiple intermediate structures – mainly in an oligomeric state – prior to the fiber formation and/or their specific interaction with membranes (Caughey and Lansbury, 2003; Haass and Selkoe, 2007). Moreover, the physicochemical properties of these intermediate structures such as size, aggregation propensity, and glycosylation state seem to correlate with the clinicopathological features (Parchi et al., 2011), thereby suggesting that $PrP^{Sc}$ fragments have variable

neurotoxicities and cause distinct lesions as a consequence of their different properties (Parchi et al., 1998).

## 5. Prion strain

Like conventional infectious agents, mammalian prions occur in a variety of different strains that are defined as natural isolates of infectious prions. Prion strains cause distinctive disease phenotypes (including incubation times, clinical signs, and the histopathological lesion profiles and $PrP^{Sc}$ deposition patterns in the brain) that are faithfully recapitulated upon serial passage within the same host genotype (Beringue et al., 2008b; Collinge and Clarke, 2007). Evidence of different prion strains was first reported in goats inoculated with a pool of scrapie-infected sheep brains. Scrapie transmission resulted in two clinically different disease phenotypes: 'scratching' or 'drowsy' (Pattison et al., 1959; Pattison and Millson, 1961).

### 5.1. Molecular basis of prion strains

The Prion Protein-Only Hypothesis postulates that the different prion disease phenotypes found in animals are due to differences in the genetic information triggered by the prion agent (Chesebro, 1998). However, prions are proteins and consequently do not harbor any genetic information. Experimental data available to date provides clear evidence that prion strains are encoded by the variety of conformations that $PrP^{Sc}$ can adopt, which can affect its biological and biochemical properties. These molecular differences are thought to be the underlying cause of the widespread pathological range of prion diseases.

The first evidence of the molecular basis of the diversity of prion strains came from a study in which scrapie-associated fibrils (SAF) from scrapie-infected mice and hamsters (ME7 and 139A in mice, and 263K in hamster) were isolated and their molecular properties compared (Kascsak et al., 1985). ME7 and 139A had different morphologies, erythrocyte sedimentation rates, and sensitivity to PK digestion from those in hamster 263K, differences that were associated with

the distinct biological and pathological properties of the prion strains. In another study, two biologically distinct strains, "hyper" (HY) and "drowsy" (DY), were distinguished after transmission of transmissible mink encephalopathy (TME) in hamsters, during which differences occurred in their $PrP^{Sc}$ sedimentation in N-lauroylsarcosine, sensitivity to PK digestion, electrophoretic mobility, and even in their immunoreactivity in the N-terminus (Bessen and Marsh, 1992). These findings indicated that, although originating from the same host, the $PrP^{Sc}$ molecules of different prion strains vary in conformation and/or composition.

Studies of yeast have provided essential information for understanding the phenomenon of prion strains. A direct correlation between the frangibility (propensity to break up) of yeast $PrP^{Sc}$ fibrils and their rate of replication have been reported (Immel et al., 2007; Tanaka et al., 2006; Tanaka et al., 2004) and extended to mammalian prions. A conformational stability assay of 30 different mammalian prion isolates revealed a linear relationship between the concentrations of guanidine hydrochloride (Gdn.HCl) required to denature 50% of $PrP^{Sc}$ molecules and incubation times. This finding suggests that low $PrP^{Sc}$ stability favors fragmentation of $PrP^{Sc}$ molecules, thereby exposing more $PrP^{Sc}$ surface able to bind to further $PrP^{C}$ and finally resulting in an increased rate of $PrP^{Sc}$ formation and shorter incubation times (Legname et al., 2006).

Besides the incubation time, prion stability has also been associated with the differential ability of prions to invade the CNS (Bett et al., 2012b). Thus, highly neuroinvasive prion strains seem to be conformationally unstable in denaturing conditions and efficiently form diffuse, non-fibrillar PrP aggregates in the CNS, which produces a rapid progression to terminal disease in mice. On the other hand, weakly neuroinvasive strains form dense, congophilic, fibrillar plaques and mice progress to terminal disease more slowly.

### 5.2. Characterization of prion strains

Prion strains can be characterized according to a number of different parameters:

-Biological properties: prion strains produce specific disease phenotypes that can be identified by their incubation periods, clinical signs, and histopathological alterations such as $PrP^{Sc}$ distribution and nature, lesion profiles (spongiosis), and tissue and cellular tropisms.

-Biochemical properties: each prion strain is associated with a specific cluster of biochemical features characterizing its $PrP^{Sc}$. Of these; the major hallmarks for prion differentiation are stability toward denaturing agents, glycosilation patterns and electrophoretic mobility after PK digestion, and resistance to proteolytic degradation. Furthermore, differences between strains in their binding affinity for copper have also been described (Wadsworth et al., 1999).

-Conformational properties: distinct prion strains may display similar patterns of protease resistance but can be distinguished by their conformations. Differences in strain conformation can be revealed by sedimentation techniques (Bessen and Marsh, 1992), light scattering (Scheibel and Lindquist, 2001), both transmission electron microscopy and atomic force microscopy for assembly (Serio et al., 2000), through structural change studies, by circular dichroism (Safar et al., 1998) and dye binding (Nilsson, 2004; Sigurdson et al., 2006; Sigurdson et al., 2007), and through mapping binding sites by conformation-dependent immunoassay (CDI) (Zou et al., 2003).

## 6. Prion transmission

Prion agents can be transmitted within or even between mammalian species. Although intra-species prion transmission is normally efficient and maintains disease features, the outcome of a cross-species transmission is unpredictable. In some cases, no TSE develops, while in others, although TSE does not develop clinically, silent/subclinical infection can occur. If transmission occurs, the TSE

agent can either remain identical to the original or evolve into a completely different prion strain as an adaptation to the new species (Bruce and Dickinson, 1987; Kimberlin et al., 1987; Kimberlin et al., 1989; Pattison, 1965; Peretz et al., 2002; Prusiner et al., 1990). In this latter case, some features of the newly generated prion are related to the original prion isolate, while others may be linked to the new host $PrP^C$ amino acid sequence or to other host factors. In this second passage, the incubation periods decrease and the biochemical and neuropathological properties of the prion isolate are stabilized. This phenomenon is known as the "**species barrier**" and despite intensive investigation it still remains largely enigmatic.

## 6.1. Factors governing prion transmission

### *6.1.1. PrP genotype*

At molecular level, the transmission barrier was initially believed to be governed entirely by the degree of PrP amino acid sequence similarity between donor and recipient species. This assumption was derived from the fact that during prion infection a highly specific physical interaction between $PrP^C$ and $PrP^{Sc}$ is required for the prion conversion. Thus, minimal changes in the $PrP^C$ amino acid sequence have a large impact on the susceptibility of a species to infection with a specific prion strain.

Many studies have supported this assumption. For example, black weasel and the mink have shown different susceptibility when inoculated with the same mink-transmissible encephalopathy isolate (Bartz et al., 1994). The $PrP^C$ primary structures of these two mustelids only differ by two amino acid residues, which could be the cause of the differing susceptibilities.

Besides interspecific variations in amino acid sequences, allelic variants in the *prnp* can affect the efficiency of prion disease transmission. In humans the susceptibility to vCJD and BSE is modulated by the $V/M_{129}$ polymorphism. Cases of vCJD disease have only been observed in people who are homozygous for the $M_{129}$ allele (Collinge and Clarke, 2007), while experiments using Tg mice

have demonstrated that the human $V_{129}$ acts as a transmission barrier to the BSE prion agent, thereby producing alterations in the disease phenotype (Wadsworth et al., 2004).

*Prnp* mutations can occur in different parts of the $PrP^{C}$ sequence, although not all influence transmission efficiency to the same degree. The amount of amino-acid overlap between infectious $PrP^{Sc}$ and endogenous $PrP^{C}$ substrate within the central region of the prion protein deeply affects the efficiency of the conversion process (Scott et al., 1993b; Scott et al., 1992; Telling et al., 1995). Above all, the β2–α2 loop region of the prion protein (residues 165–175 in the mouse prion protein) has been proposed as a critical region in the $PrP^{C}$ structure since minimal changes in its amino-acid sequence seriously influence mouse susceptibility to sheep scrapie, BSE, mouse-adapted scrapie, deer chronic wasting disease, and hamster-adapted scrapie prions (Bett et al., 2012a; Sigurdson et al., 2011; Sigurdson et al., 2010).

More recently, bank vole $PrP^{C}$ was proposed as a universal acceptor for prions (Watts et al., 2014) since Tg mice overexpressing this species' $PrP^{C}$ are susceptible to a wide range of prion isolates from many different species (including humans, cattle, elk, sheep, guinea pigs, hamsters, mice, and meadow voles). This exceptional promiscuity of bank vole PrP Tg mice further indicates that only a small number of amino acid differences in the PrP influence the conversion efficiency of prions.

### *6.1.2. Prion strain*

Transmission efficiency is also profoundly determined by the prion strain. For example, bovinized Tg mice PrP can be infected with vCJD at ~270 days but need over 500 days to succumb to sCJD subtype MM1 (Giles et al., 2010; Scott et al., 2005; Scott et al., 1999). Notably, the prion agents used in these transmission studies (vCJD and sCJD subtype MM1) harbored the same amino acid sequence, thereby supporting the idea that the differences in the sequences of $PrP^{Sc}$ in the inoculum and $PrP^{C}$ in the host are insufficient to explain all aspects

of prion transmission from one host to another. Similarly, Tg mice expressing porcine PrP are susceptible to sheep-BSE but not to scrapie (Espinosa et al., 2009).

Recently, our group has compared the properties of BSE prions after transmission to different species with other non-BSE related prions, and has shown that the BSE agent was able to cross the trans-species barrier without any fundamental alteration of their strain specific features; on the other hand, a high species barrier was observed for non-BSE related prions harboring an identical PrP amino acid sequence (Torres et al., 2014). These results confirm that BSE is a highly promiscuous prion agent and supports the contention that prion transmission barriers might be modulated by strain properties (presumably conformation-dependent) rather than by PrP amino acid sequence differences existing between host and donor.

### 6.2. Emergence of prion strains: "adaptation" vs. "selection"

The emergence of new prion strains occurs when a propagated prion does not maintain the same biochemical and pathogenic characteristics as the original strain. This phenomenon can occur either during cross-species transmission or within a single species (Falsig et al., 2008). In the first case, the sequence of the $PrP^C$ of the host is different from that of the donor (prion agent) and, due to the species barrier, the conversion process can lead to a different strain (Collinge et al., 1996). In the second case, the *prnp* gene, coding for the $PrP^C$, is identical in the donor and the host, suggesting that other mechanisms such as cofactors or host cellular environment or polymorphisms of the $PrP^C$ sequence (see below) may be involved in strain selection.

Two theories have been proposed to explain the emergence of prion strains: strain **"mutation"** and strain **"selection"**. The former postulates that strains exist as a single clone that can suffer a change into one or multiple new strains after transmission into a host (Fig. 6B2). This shift results in new biochemical, biological, or histological properties. By contrast, the latter theory proposes that

strains exist as a mixture of $PrP^{Sc}$ conformers in a relative proportion, which varies upon transmission to different species (Angers et al., 2010). This process would lead to the emergence of new strain properties but not to the emergence of new prion strains (Fig. 6D).

### 6.3. Conformational Selection model

The "conformational selection" model was postulated to explain how the host $PrP^{C}$ sequence influences the ability to propagate strain features. This model proposes that a prion strain would consist of a cloud of possible $PrP^{Sc}$ conformations, i.e. a dominant $PrP^{Sc}$ species plus other less abundant $PrP^{Sc}$ structures, and that its transmission would be determined by the degree of overlap between the range of $PrP^{Sc}$ conformers that the prion strain harbors and the conformational constraints imposed by the sequence and conformation of the host $PrP^{C}$ (Fig. 6D) (Beringue et al., 2008b; Collinge and Clarke, 2007).
According to this model, depending on their $PrP^{C}$ properties and the $PrP^{Sc}$ conformations in the infecting inoculum, each mammal species could be infected by a range of possible $PrP^{Sc}$ conformations. In this way, the relative composition of this cloud is maintained in its original host but upon passage to a new host it may adopt a different ratio of conformations and thus a new strain is formed (Fig. 6D). Therefore, the emergence of the new strain would result from an inability of the new host $PrP^{C}$ sequence to adopt the dominant $PrP^{Sc}$ structure and so a different subset of $PrP^{Sc}$ conformers are propagated instead.

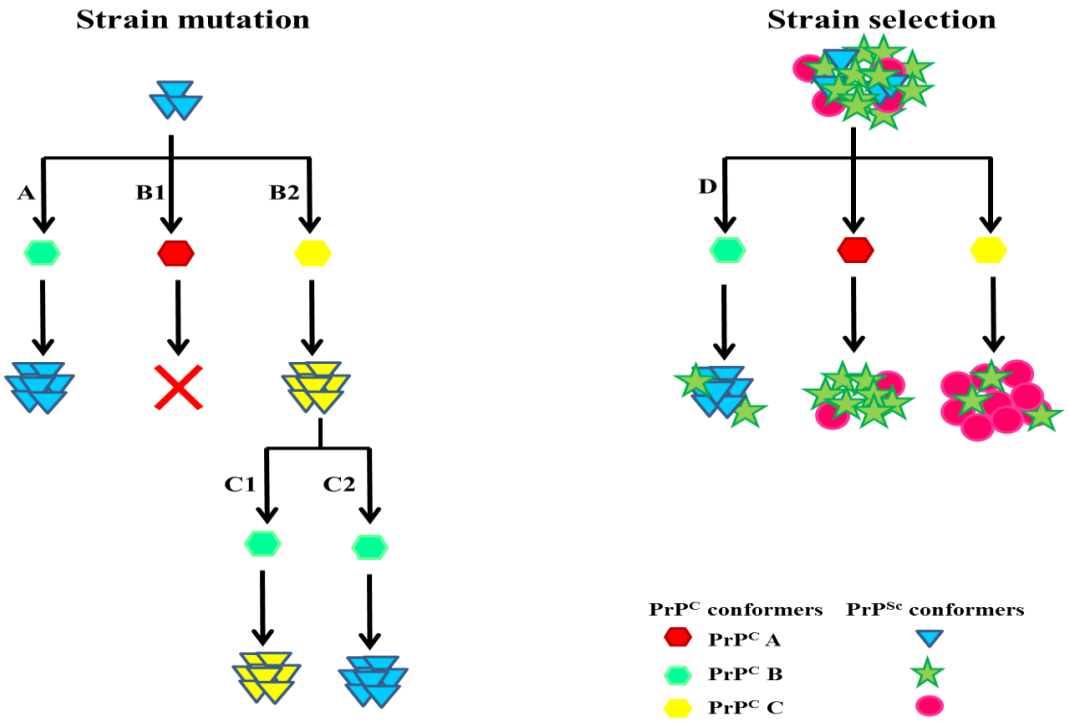


**Figure 6. Models for the emergence of prion strains and transmission barrier.** The transmission barrier is determined by the degree of overlap between the range of $PrP^{Sc}$ conformers (represented by different shapes: stars, triangles, and circles) and the diverse sequences of the $PrP^{C}$ in the host (represented by different colors) (adapted from (Beringue et al., 2008b; Collinge and Clarke, 2007). Several possibilities may occur:

A. The infection of $PrP^{C}$ A with a prion strain from its species resulting in the propagation of the original phenotypic properties of the strain.
B. The heterologous transmission of the prion strain from species A may result in two different outcomes. $PrP^{C}$ B may not be able to adopt the conformation of the prion strain from species A and so transmission is not possible (B1). Conversely, $PrP^{C}$ C can replicate this prion strain, although its properties are modified by a phenomenon known as mutation (B2).
C. Further passage of the newly formed strain may result in the maintenance of the properties formed during previous passage (C1). Nevertheless, transmission to the $PrP^{C}$ species of origin may rescue the original properties of the prion strain from species A (C2).
D. Conformational selection: Strains harbor a range of conformations that interact with $PrP^{C}$. Only the most compatible $PrP^{Sc}$ conformations with the conformational constraints imposed by the host $PrP^{C}$ sequence are replicated by the $PrP^{C}$ species, thereby giving rise to a variety of strain characteristics.

Alternatively, multiple $PrP^{Sc}$ types might be produced *de novo* during heterologous transmission (C). Independently of the mechanism, mutation, or selection, the establishment of the new strain properties might be gradual upon serial transmissions within the host.

The molecular mechanism by which the range of $PrP^{Sc}$ conformers is produced and selected during the process of strain "adaptation" or "selection" is as yet unknown. One possibility is that each $PrP^{Sc}$ conformer requires a unique set of cofactors to propagate efficiently, and that the distribution of these cofactor molecules varies between animal species and even between cell types. In line with this view, different cell types within the same host can offer unique environments and selective pressures, each resulting in the emergence of different mutants as major constituents of the evolving population (Aguzzi and Sigurdson, 2004; Li et al., 2010; Mahal et al., 2007; Tremblay et al., 2004). Lymphotropism is a prion characteristic that varies greatly depending on the strain (Bartz et al., 2005). For example, scrapie, CWD, and vCJD are able to replicate in lymphoid tissues, while cattle BSE agent is only poorly lymphotropic but increases this ability when adapted to sheep PrP sequence (reviewed in (Beringue et al., 2008b)).

Selective metal binding could be another mechanism of prion strain generation. In an *in vitro* conversion study performed with two different human prion strains isolated from two clinically different cases of sCJD, one sCJD strain could have converted into the other merely by changing its metal ion occupancy, thereby indicating that copper and zinc binding can influence $PrP^{Sc}$ conformation (Wadsworth et al., 1999).

## 7. Human transmissible spongiform encephalopathies

Human prion diseases are classified according to their origin as sporadic, familial, or inherited, and as infectious or acquired. Polymorphism M/V at codon 129 of the human *prnp* appears to be a key factor in human prion susceptibility and to act as a modifier of the disease phenotype in human prion diseases (Alperovitch et al., 1999; Collinge et al., 1991; Doh-ura et al., 1991; Palmer et al., 1991; Zeidler et al., 1997). In Caucasians, 52% of individuals are M homozygous (MM), 36% are heterozygous (MV), and 12% are V homozygous (VV) (Alperovitch et al., 1999; Collinge and Palmer, 1994). Approximately 95% of the sCJD patients who carry $M/M_{129}$ *prnp* genotype develop a $PrP^{Sc}$ type 1 or sCJD, whereas 86% of the patients harboring either the $V/V_{129}$ or $M/V_{129}$ develop a $PrP^{Sc}$ type 2 sCJD or ataxic sCJD (Parchi et al., 1996; Parchi et al., 1999). Furthermore, $M/M_{129}$ patients who harbor the $N/D_{178}$ mutation develop a Fatal Familial Insommia (FFI)-like syndrome, while $V/V_{129}$ patients develop a familial CJD (Lloyd et al., 2011). In familial CJD cases, the codon 129 polymorphism seems to modulate the age of onset and duration of the illness (Gambetti et al., 1995).

### 7.1. Inherited transmissible spongiform encephalopathies

Familial CJD forms comprise 10–15% of all human prion diseases. All these forms are inherited as autosomal dominant traits and most are linked to point mutations in the ORF of the *prnp* gene, including 24 missense point mutations, 27 octapeptide repeat mutations with insertions of 1, 2, and 4–9 additional repeats, 2 octapeptide repeat mutations with deletion of two repeats, and two nonsense mutations (Fig. 7). Most are located at the C-terminal domain on α2 and α3 helices. Each mutation has been linked with a specific disease phenotype and familial CJD cases are commonly classified as one of three phenotypes: Gerstmann-Sträussler-Scheinker disease (GSS), familial CJD (fCJD), and fatal familial insomnia (FFI).

GSS has an average age of onset of 50–60 years and is characterized by a slowly progressive cerebellar dysfunction, ataxia, nystagmus, dysarthria, and cognitive decline. GSS has been associated with numerous mutations but mainly with $P/L_{102}$ (Glatzel et al., 2005; Kovacs and Budka, 2009; Piccardo et al., 1998). fCJD presents very similar clinical features to sCJD and *prnp* sequencing is required to guarantee correct identification (Glatzel et al., 2005). FFI has an average age of onset of around 49 years and can be associated with the $D/N_{178}$ mutation in combination with $M/M_{129}$. It causes sleep disturbance, oneiric or stuporous episodes characterized by hallucinations and confusion, autonomic dysfunction, and often myoclonus, spasticity, and seizures (Glatzel et al., 2005; Kovacs and Budka, 2009).

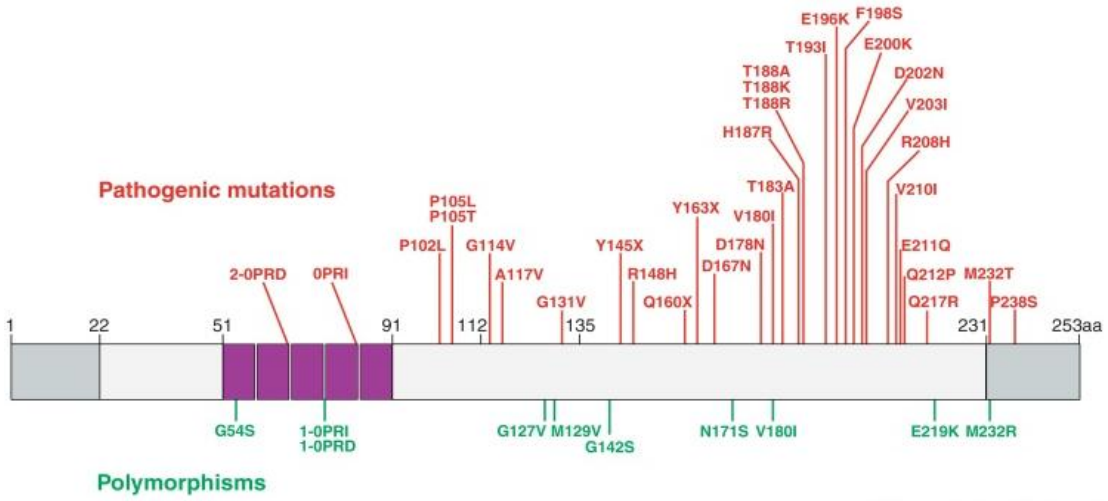


**Figure 7. PrP mutations and polymorphisms of human $PrP^C$.** Schematic representation of full-length human $PrP^C$, including disease-associated mutations (in red) and non-synonymous non-pathogenic genetic variants (in green). (OPRD: octapeptide repeat deletion; OPRI: octapeptide repeat insertion). Cleaved signal sequences (in grey) and octapeptide repeat region (in purple) are shown (extracted from (Lloyd et al., 2013).

## 7.2. Sporadic transmissible spongiform encephalopathies

sCJD represents ≈85% of all CJD cases (Masters et al., 1979) and occurs at a rate of 1–2 cases/million population (Mead et al., 2003). sCJD has a peak age of onset at 55–75 years (Glatzel et al., 2005), although some younger (under 20 years) and older (over 90 years) cases have been reported. The etiology of sCJD is unknown. The transmission of human or animal prions, the somatic mutation of the *prnp*, or spontaneous $PrP^{C}$ into $PrP^{Sc}$ conversion have all been proposed as possible origins (Prusiner, 1998a).

The disease is characterized by rapidly progressive dementia, cerebellar dysfunction including muscle incoordination, and visual, speech, and gait abnormalities, spontaneous or induced mioclonia, extrapyramidal or pyramidal dysfunction with reflexes, tremors, spasticity, and rigidity, behavioral alterations such as agitation, confusion, and depression, and a state of akinetic mutism at the end of the course of the disease (Belay, 1999). The illness usually lasts 3–6 months (Glatzel et al., 2005; Kovacs and Budka, 2009).

The clinicopathological and molecular phenotypes of sCJD are profoundly influenced by mutations in the *prnp* genotype. In this way, $M_{129}$ homozygotes account for approximately 70% of all sCJD cases (Kovacs and Budka, 2009). Current classifications of CJD are mainly based on the combination of genotype (polymorphism at codon 129 of *prnp*) and $PrP^{res}$ molecular characteristics (Collinge et al., 1996; Hill and Collinge, 2003Head, 2004 #966; Parchi et al., 1999; Wadsworth et al., 1999; Zanusso et al., 2001).

## 7.3. Infectious transmissible spongiform encephalopathies

### *7.3.1 Kuru*

Kuru is a neurological disorder endemic to the Fore linguistic tribal regions of Papua New Guinea (Gajdusek and Zigas, 1957). The term "kuru" derives from the Fore word *kuria/guria* ("to shake") and refers to the body tremors exhibited by patients. The clinical stage lasts for between 3 months to 2 years

(on average of 12 months) and has three clearly different stages: ambulant (infected people can still walk), sedentary (can only sit up), and terminal (unable to sit up independently). Cerebellar ataxia, tremors, deterioration of speech, and dysarthria are other distinctive clinical signs. Kuru was the first human TSE transmitted to chimpanzees (Gajdusek et al., 1967). The etiology of the disease was soon linked to endocannibalistic funeral practices and, after a ban on ritualistic cannibalism imposed by the Australian authorities in the mid-1950s, the incidence of the disease started to decline steadily (Alpers, 2008).

### *7.3.2 Iatrogenic CJD (iCJD)*

Iatrogenic transmission of CJD occurs due to the use of stereotactic intracerebral electrodes or neurosurgical instruments that have been inadequately sterilized, cadaveric dura mater grafts, or intramuscular injections of contaminated cadaveric pituitary-derived human growth hormone (hGH) and gonadotrophin hormone. Clinical signs are similar to sCJD, although in some cases due to hormone treatment cerebellar symptomatology may dominate (Brown et al., 1992; Glatzel et al., 2005; Kovacs and Budka, 2009).

### *7.3.3 Variant CJD*

The first variant CJD (vCJD) cases were reported in 1996 in UK in teenagers and young adults with psychiatric symptoms instead of the cerebellar ataxia or progressive dementia that is typical of CJD (Will et al., 1996). Soon after the first reports of these cases, epidemiological studies along with the experimental transmission of the disease to cynomologous macaques and mice and biochemical strain-typing managed to link the etiology of vCJD to exposure to BSE-infected meat products (Bruce et al., 1997; Hill et al., 1997; Will et al., 1996). As of June 2014, 226 vCJD diagnoses have been reported worldwide, most of them in the UK (Table 2) (http://www.cjd.ed.ac.uk/documents/worldfigs.pdf). Once again, $M/V_{129}$ human polymorphism has a determinant influence on vCJD cases and, to date,

all except one clinical case have been homozygous M/$M_{129}$ (Kaski et al., 2009; Kovacs and Budka, 2009).

The psychiatric symptoms of vCJD include agitation, aggression, depression, anxiety, apathy, sensory disturbance (paresthesia, diesthesia, and pain), and paranoid delusions. Neurologic symptoms begin at least six months after the onset of psychiatric symptoms and comprise cerebellar ataxia, cognitive impairment, and involuntary movements that may be dystonic, choreiform, or myoclonic (Spencer et al., 2002). The mean age at the onset of symptoms is 29 years, and the total duration of the disease is on average 18 months (Belay, 1999; Will, 2003). Neuropathological hallmarks of vCJD are florid plaques that predominantly occur in the cerebellum and cerebrum (Belay, 1999). vCJD $PrP^{res}$ glycoprofile is similar to that observed in cattle BSE but is different from that observed in sCJD (Collinge et al., 1996; Hill et al., 1997).

Importantly, at least four cases of vCJD in UK have been associated with secondary human-to-human transmission due to blood transfusions (Wroe et al., 2006), which has raised serious public health concerns (Hewitt et al., 2006; Llewelyn et al., 2004; Sibbald, 2004). As a consequence, in 2004 the UK Blood Service banned transfusion recipients from donating blood.

**Table 2.** vCJD cases worldwide (June 2014)

| Country | Total number of primary cases | Total number of secondary cases | Residence in UK >6 months during 1980-1996 |
|---|---|---|---|
| UK | 174 | 4 | 177 |
| France | 27 | - | 1 |
| Spain | 5 | - | 0 |
| Republic of Ireland | 4 | - | 2 |
| USA | 4 | - | 2 |
| Netherlands | 3 | - | 0 |
| Canada | 2 | - | 1 |
| Italy | 2 | - | 0 |
| Portugal | 2 | - | 0 |
| Japan | 1 | - | 0 |
| Saudi Arabia | 1 | - | 0 |
| Taiwan | 1 | - | 1 |

## 8. Transmissible spongiform encephalopathies in animals

### 8.1 Scrapie

#### *8.1.1. Classical scrapie*

Scrapie is a TSE naturally affecting sheep, goats, and mouflons (Jeffrey and Gonzalez, 2007) that is today endemic in many countries worldwide. Scrapie is characterized by its long incubation periods (2–5 years) and survival times ranging from two weeks to six months. Clinical signs include behavioral changes (fixed stare, isolation, hyperexcitability, loss of inquisitiveness), trembling, incoordination of gait, weight loss or emaciation, pruritus (the main symptom in sheep, which usually leads to wool loss), and impaired vision (Bellworthy et al., 2008; Dickinson, 1976; Hadlow et al., 1982).

Associated neurological lesions depend deeply on the scrapie strain but generally include neuronal degeneration, non-inflammatory spongiform changes, and astrogliosis, which are detected mainly in the diencephalon, midbrain, pons, medulla oblongata, and cerebellar cortex (Hadlow et al., 1982). Apart from the nervous system, $PrP^{Sc}$ deposition has also been observed in the tonsils (Andreoletti et al., 2000), spleen (Hadlow et al., 1982), lymph nodes (van Keulen et al., 2008a), nicitating membrane, muscles, placentas (Andreoletti et al., 2002), skin (Garza et al., 2014; Thomzig et al., 2007), mammary glands (Ligios et al., 2005), distal ilium, proximal colon (van Keulen et al., 2008a), and, more recently, in the pancreas, heart, and urinary bladder (Garza et al., 2014).

Although there is some evidence of vertical transmission (Spiropoulos et al., 2014), the most likely route of prion infection seems to be contact transmission between ewes and their lambs around the time of birth (Imran and Mahmood, 2011). The presence of scrapie infectivity in blood (Bannach et al., 2012; Dassanayake et al., 2012; Dassanayake et al., 2011; Lacroux et al., 2012), saliva (Gough et al., 2011; Tamguney et al., 2012), milk (Ligios et al., 2011), and colostum (Konold et al., 2013) in conjunction with the high resistance of this prion agent against denaturizing factors contribute to its permanence in the

environment, i.e. in the soil (Saunders et al., 2012b), and consequently favors horizontal transmission within sheep and goats herds.

To date, epidemiological and transmission studies have failed to demonstrate any link between scrapie disease and any human prion disease despite worldwide endemicity ((BIOHAZ), 2011; Wadsworth et al., 2013). More recently, some classical scrapie isolates from infected sheep have been transmitted to humanized Tg mice expressing either the $M_{129}$ or $V_{129}$ polymorhic variants with similar efficacy as in cattle-BSE (Lacroux et al., 2014b). Interestingly, after serial transmission in these humanized Tg mice, the scrapie isolates were phenotypically identical to either sCJD type 1 or sCJD type 2. These results demonstrate the zoonotic potential of classical scrapie agents and are an argument for their prompt control and eradication.

### *8.1.2 Scrapie strains: classical scrapie vs. atypical scrapie*

Increased surveillance over the past two decades has led to the identification of a wide variety of scrapie disease phenotypes, which differ in terms of their incubation periods, clinical signs, $PrP^{res}$ WB profile, $PrP^{Sc}$ distribution, and pathogenesis. These differences are suggestive of a number of scrapie strains and highlight the tremendous heterogeneity of the scrapie disease. In addition, an unusual type of scrapie was identified in 1998 in Norway and was named atypical scrapie Nor-98 (Benestad et al., 2003). Currently, an increasing number of atypical/Nor98 scrapie cases are being reported from many European countries, as well as from the USA (Benestad et al., 2008) and New Zealand (Kittelberger et al., 2010). Its clinical signs are similar to classical scrapie disease, albeit are generally less pronounced. Pruritus is uncommon and the major clinical symptoms are ataxia and incoordination (Imran and Mahmood, 2011). Unlike classical scrapie, the $PrP^{Sc}$ deposition pattern in atypical scrapie infections is mild and restricted to the obex but is more intense throughout the cerebellum, substantia nigra, thalamus, and basal nuclei (Moore et al., 2008).

Despite the increasing number of atypical scrapie cases, no evidence of strains have appeared so far for this TSE and so no major differences have been determined in incubation periods, clinical signs, $PrP^{res}$ WB profile, $PrP^{Sc}$ distribution, or pathogenesis in the reported natural and experimental cases (Gotte et al., 2011; Griffiths et al., 2010; Le Dur et al., 2005; Pirisinu et al., 2010; Pirisinu et al., 2013). This fact clearly contrasts with the high heterogeneity registered for classical scrapie and further supports the contention that atypical scrapie is a homogenous entity (Gotte et al., 2011; Griffiths et al., 2010; Le Dur et al., 2005; Pirisinu et al., 2010; Pirisinu et al., 2013).

On the other hand, atypical scrapie has been proposed as spontaneous in origin since is quite widespread and often occurs in older animals as single cases in a whole flock (Benestad et al., 2008; Fediaevsky et al., 2010; Hopp et al., 2006). Nevertheless, some findings have demonstrated the oral transmissibility of the atypical scrapie agent (Simmons et al., 2007; Simmons et al., 2011) and this fact, together with the presence of prion infectivity in different tissues (including lymphoid tissues, nerves, and muscles (Andreoletti et al., 2011; Simmons et al., 2011)), seems to indicate that natural transmission may be possible in atypical scrapie.

### *8.1.3 Factors determining scrapie occurrence*

Scrapie occurrence is mainly determined by the host prion protein encoding gene (*prnp*) (Agrimi et al., 2003; Baylis et al., 2004) and the prion strain (Aguzzi et al., 2007) and thus results in distinct disease phenotypes with differences in the incubation period, pathogenesis, clinical signs $PrP^{res}$ WB profile, and $PrP^{Sc}$ deposition pattern.

Sheep and goats share the same $PrP^{C}$ amino acid sequence but with a variety of polymorphism (Fig. 8). Sheep *prnp* genotypes $V_{136}R_{154}Q_{171}$ and $A_{136}R_{154}Q_{171}$ are associated with a high susceptibility to classical scrapie, while the $A_{136}R_{154}R_{171}$ genotype is linked to resistance (Belt et al., 1995; Bossers et al., 1996; Hunter, 1996, 1997). Interestingly, atypical scrapie more frequently affects sheep

carrying resistant genotypes (such as $A_{136}R_{154}Q_{171}$, $A_{136}H_{154}Q_{171}$) than susceptible animals (i.e. $V_{136}R_{154}Q_{171}$). Indeed, atypical scrapie seems to be strongly modulated by $R/H_{154}$ and $F/L_{141}$ polymorphisms (reviewed in (Benestad et al., 2008)).

In goats, around 50 polymorphisms in the open reading frame (ORF) of the *prnp* gene have been described (Acin et al., 2013; Fragkiadaki et al., 2011; Vaccari et al., 2009a), including some silent mutations, a three octapeptide repeats variant (Goldmann et al., 1998), and a nonsense mutation at codon 32 (Benestad et al.) (Fig. 8). Although several polymorphisms are shared by sheep and goats, to date only some correlations between variants of the goat $PrP^C$ and susceptibility to TSE have been published.

The $I/M_{142}$ amino acid substitution is associated with a slightly lower risk of developing scrapie (Barillet et al., 2009; Goldmann et al., 2011; Gonzalez et al., 2010) and prolonged incubation times after challenges from scrapie and BSE prions (Goldmann et al., 1996). In natural outbreaks, goats carrying the $H/R_{143}$ polymorphism showed a partial decrease in their susceptibility to scrapie prions (Barillet et al., 2009; Billinis et al., 2002). Likewise, a low susceptibility to scrapie has also been linked to the $R/H_{154}$ and $R/Q_{211}$ variants of the goat $PrP^C$ (Barillet et al., 2009; Bouzalas et al., 2010), although the $R/H_{154}$ polymorphism also arose as a risk factor for goat atypical scrapie (Colussi et al., 2008). Other $PrP^C$ variants such as the $N/D_{146}$ and $N/S_{146}$ polymorphisms have been linked to resistance to scrapie (Papasavva-Stylianou et al., 2011).

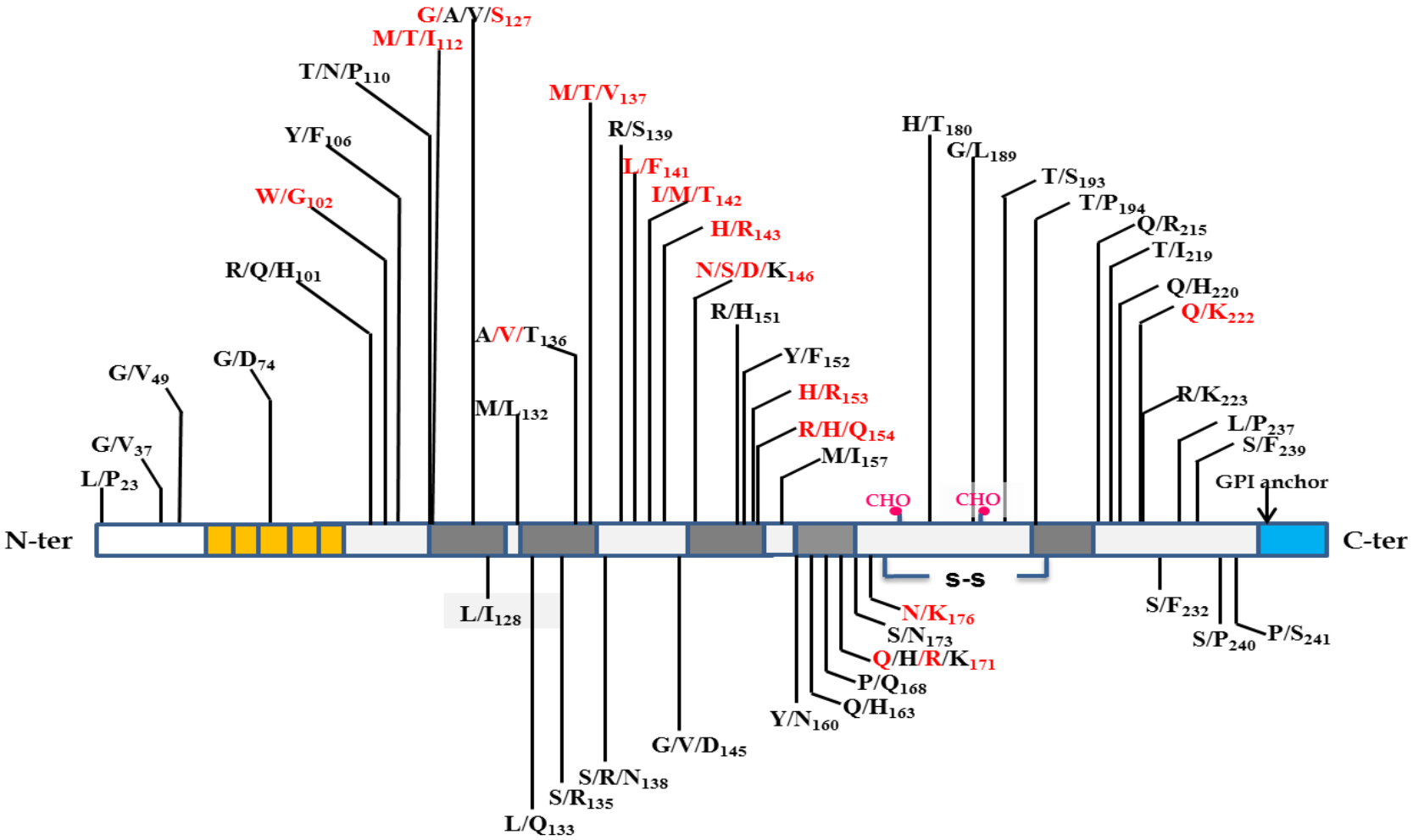


**Figure 8. PrP polymorphisms of sheep and goat PrP$^C$.** A schematic representation of full-length sheep/goat PrP$^C$ including all the PrP polymorphisms reported and their effect on increasing resistance (in red) to classical scrapie.

However, the most encouraging results are those related to the $Q/K_{222}$ polymorphism. The absence or marked under-representation of scrapie-positive goats expressing the $K_{222}$ variant in herds from many different countries (Acin et al., 2013; Acutis et al., 2006; Barillet et al., 2009; Bouzalas et al., 2010; Corbiere et al., ; Fragkiadaki et al., 2011; Papasavva-Stylianou et al., 2011; Vaccari et al., 2006) suggests that the $K_{222}$- $PrP^C$ variant strongly influences goat susceptibility to TSE agents.

Most of these associations have been determined as a result of the intense epidemiological surveillance program for TSE in small ruminants implemented by the European Union (EU) in 2002, which has subsequently been adopted by non-EU countries such as Canada and the USA. This program consists of both passive and active surveillance through post-mortem testing of a fraction of small ruminants at abattoirs or rendering plants (Corbiere et al., 2013a). Currently, scrapie control regulations in the EU consist of 1) culling all animals of a susceptible genotype in infected flocks (if no genetic testing is carried out, all animals are culled) and 2) the genetic testing of rams intended for breeding in scrapie-free flocks of "high genetic merit", followed by the culling of the susceptible rams (EC Regulation No 999/2001).

This system provides an estimate of scrapie occurrence in the EU and enables both the trend of classical scrapie evolution in populations over the past decade and the genotypes governing susceptibility to scrapie to be monitored. Although this epidemiological data is highly valuable for controlling scrapie, experimental confirmation is still necessary to obtain conclusive results.

### *8.1.4 Selective breeding programs*

Epidemiological studies provide highly valuable information about the potential role of certain polymorphic variants of goat and sheep $PrP^C$ and these animals' susceptibility to both classical and atypical scrapie. This work is normally based on case-control studies. Unfortunately, the low frequency of

certain polymorphisms in goat and sheep populations prevents us from drawing conclusions regarding their effect on susceptibility to prion infection.

Experimental studies in sheep have demonstrated a clear link between the $A_{136}R_{154}R_{171}$ genotype and resistance to classical scrapie (Belt et al., 1995; Bossers et al., 1996; Hunter, 1996, 1997). On this basis, selective breeding programs have been implemented in some EU member states in order to promote the $A_{136}R_{154}R_{171}$ haplotype within ovine herds (Dawson et al., 1998). This strategy has resulted in the rapid control of scrapie outbreaks (Nodelijk et al., 2011) and has lowered the risk of scrapie infection even in animals with susceptible genotypes (Hagenaars et al., 2010; Kanata et al., 2014).

Unfortunately, atypical scrapie is more common in genotypes such as $A_{136}R_{154}R_{171}$ and $A_{136}H_{154}R_{171}$ sheep than in genotypes associated with high susceptibility to classical scrapie such as $V_{136}R_{154}Q_{171}$ (Benestad et al., 2008). Hence, selective breeding programs could have indirectly favored the incidence of atypical scrapie in sheep herds, which has prompted a search for new resistant genotypes to control and eradicate both classical and atypical scrapie in sheep herds.

Epidemiological and *in vitro* studies are of great use in this task. However, results must be confirmed by experimental inoculations in sheep and goats before drawing definitive conclusions. Due to the high number of *prnp* genotypes, their low frequencies in sheep and goat populations, and the unknown number of scrapie strains currently in circulation, experimental challenges in sheep and goats are not feasible. In this sense, Tg mice may be a good alternative. The generation of Tg mice, each expressing either a sheep or goat $PrP^C$ variant, would help clarify their role in scrapie and BSE susceptibility and decide which is the most suitable for breeding programs.

### 8.2. Bovine Spongiform Encephalopathy (BSE)

BSE, commonly known as "Mad Cow Disease", was firstly diagnosed in UK in 1986 (Wells et al., 1987). It soon spread worldwide and became epidemic. BSE-infected cattle have incubation periods of over two years and clinical signs including cachexia, alopecia, apprehension, lethargic or aggressive behavior, hyper-responsiveness to stimuli, and abnormalities in movement (i.e. ataxia, particularly of the hind-limbs).

Histolopathological lesions consist of vacuolation, predominantly in the medulla oblongata at the level of the obex (Jeffrey and Gonzalez, 2004), and also in the central grey matter, rostral colliculus, and hypothalamus (Simmons et al., 1996; Wells et al., 2005). Unlike scrapie, $PrP^{Sc}$ deposition in BSE-infected animals is mostly confined to the nervous system. However, low infectivity in Peyer's patches of the small intestine, distal ileum, jejunum, ileocecal juction (Hoffmann et al., 2011), and tonsils (Buschmann and Groschup, 2005; Espinosa et al., 2007b; Hoffmann et al., 2011; Stack et al., 2011; Terry et al., 2003; Wells et al., 2005) have also been described.

More recently, BSE infectivity in skeletal muscles has been determined and associated with a probable centrifugal spread of the agent from central nervous tissues through the somatic motor and/or sensory pathways to peripheral muscle tissues (Okada et al., 2014). This observation is extremely relevant for redefining the Specified Risk Materials as stated by the Food Standards Agency to prevent the entry of BSE-contaminated material into the foodchain.

To date, there is no evidence of BSE infectivity in semen, embryos, placenta, or milk, (Bradley and Wilesmith, 1993; Buschmann and Groschup, 2005; Taylor et al., 1995; Wrathall et al., 2002), although an increased risk of BSE development has been reported in the offspring of infected cows. The practice of feeding cattle with meat and bone meal (MBM) contaminated with infectious prions was initially proposed as the most likely cause of the BSE epidemic (Wilesmith et al., 1991). A number of hypotheses regarding the origin of BSE have been

considered, including i) the primary existence of sporadic or genetic BSE in cattle before its transmission via MBM (Baron et al., 2011; Capobianco et al., 2007; Nicholson et al., 2008; Richt and Hall, 2008; Torres et al., 2013); ii) sheep or goat-scrapie transmission to cattle through MBM (Hill et al., 1998), and iii) human CJD (Colchester and Colchester, 2005). The European ban on the feeding of MBM to ruminants has sharply decreased the incidence of the disease, although it has still not been completely eradicated.

The BSE agent has demonstrated a high capacity to cross species barriers. During the BSE epidemic in the 1980s, the disease spread not only to humans with the emergence of variant Creutzfeldt-Jacob disease (vCJD) (Bruce et al., 1997; Hill et al., 1997) but also to cats and a variety of zoo animals, which probably gave rise to Feline Spongiform Encephalopathy (FSE), Exotic Ungulate Spongiform Encephalopathy (EUE), and TSE in non-human primates (NHP) (reviewed in (Sigurdson and Miller, 2003)). Furthermore, BSE has been experimentally transmitted with great efficiency to mice, hamsters, sheep, goats, pigs, mink, and non-human primates (Brown et al., 2003; Holznagel et al., 2013; Hunter, 2003; Lasmezas et al., 2005; Wells et al., 2003).

Upon passage in sheep, BSE becomes more lymphotropic (Foster et al., 2001b) but also increases its transmissibility to humans (Padilla et al., 2011; Plinston et al., 2011; Priem et al., 2014). These facts have important implications for public health, especially after two "natural" BSE cases in goats were reported (Eloit et al., 2005; Jeffrey et al., 2006). Consequently, political regulations were changed to prioritize biochemical differentiation between BSE and scrapie agents in sheep and goats (European and Council, 2005). Several biochemical tests have been approved for differentiating BSE and scrapie, while immunohistochemical procedures have been established with the same purpose for lymphoid tissues (Thuring et al., 2005) and the central nervous system (Siso et al., 2010).

Unlike scrapie, little is known about the factors determining the susceptibility of goats and sheep to BSE. Some authors report prolonged incubation times in BSE-infected goats harboring the $I/M_{142}$ polymorphism (Goldmann et al., 1996),

although $L/F_{141}$ polymorphism seems to produce the same effect in BSE-infected sheep (Tan et al., 2012). It has also been demonstrated that the ARR/ARR genotype (promoted by selective breeding programs for controlling/eradicating scrapie in sheep populations) does not protect sheep against BSE infection (Andreoletti et al., 2006; Bencsik and Baron, 2007). These findings support the view that sheep and goat-BSE are new potential risks for human health and that strategies for their control are lacking.

### *8.2.1 Atypical BSE*

In recent years, active surveillance of BSE in cattle has led to the discovery of two "atypical" BSE variants named H-type (Biacabe et al., 2004) and L-type BSE (Casalone et al., 2004). These variants are often detected in fallen cattle and slaughtered old animals, and differ from classical BSE in their biochemical properties and histopathological lesions. Their low prevalence worldwide is consistent with a sporadic origin. However, their experimental transmission to bovinized, ovinized, and wild type mice suggest a potentially infectious nature (Beringue et al., 2007; Buschmann et al., 2006; Capobianco et al., 2007; Torres et al., 2011). Unlike the classical BSE agent, several changes occur in the biological and biochemical properties of atypical BSE variants when transmitted to other species. Many of these changes result in the emergence of classical BSE features, which suggests a possible relationship between atypical BSE variants and the origin of the BSE epidemic (Beringue et al., 2007; Beringue et al., 2006; Capobianco et al., 2007; Torres et al., 2011).

Although no epidemiological link between atypical BSE and any human prion disease has yet been determined, it has been reported that the L-type BSE agent can propagate into humanized mice overexpressing the $M_{129}$-$PrP^C$ variant without any significant transmission barrier, and is even more infectious for humans than epidemic classical BSE (Beringue et al., 2008a). Similar outcomes have been obtained in non-human primates, in which the L-type BSE agent is a noted zoonotic risk (Comoy et al., 2008; Ono et al., 2011). Conversely,

transmission studies have failed to transmit H-type BSE to humanized mice (Beringue et al., 2008a; Wilson et al., 2012).

### 8.3 Chronic Wasting Disease (CWD)

CWD is a naturally occurring TSE affecting farmed and free-ranging cervids. Its main symptoms are marked weight loss, rough dry coat, excessive salivation, pacing, sudden death after handling, lowered head and drooping ears, and behavioral changes such as a loss of fear of humans. Survival times range from seven to eight months in deer (Williams and Young, 1980) but can be longer in elks (Miller et al., 1998). In CWD-affected animals, $PrP^{Sc}$ can be widely distributed throughout the nervous system but also in the lymphoreticular and hematopoietic systems, pancreas, muscles, fat, retina, placenta, and the adrenal and salivary glands (Race et al., 2009a; Seelig et al., 2010; Sigurdson, 2008; Sigurdson and Miller, 2003; Spraker et al., 2010). It is unknown whether CWD arises spontaneously or was transmitted from other species. Scrapie would be a possible origin since the intracerebral inoculation of elks with this agent has induced CWD features (Hamir et al., 2004).

Since the first case was recorded in 1967 in Colorado (Williams and Young, 1980), CWD has spread unchecked throughout at least 15 states in the USA and two Canadian provinces (Saunders et al., 2012a), and has even reached South Korea due to the importation of CWD-infected animals. Horizontal transmission is the most likely infection route. It can be efficiently transmitted by contact with affected animals since CWD prions are secreted and excreted in urine, feces, saliva, and blood, or through environmental exposure to CWD-contaminated graze, soil, and water (Almberg et al., 2011; Kuznetsova et al., 2014; Mathiason et al., 2006; Miller et al., 2004; Nichols et al., 2009; Safar et al., 2008; Sigurdson, 2008; Sigurdson and Miller, 2003; Tamguney et al., 2009). Limited maternal transmission has also been proposed (Nalls et al., 2013).

In contrast to its high transmissibility among cervids, to date no natural transmission to any other species has been described. Moreover, it has been

experimentally determined that CWD is unable to cross the species barrier via the oral route (Sigurdson, 2008; Sigurdson and Miller, 2003; Tamguney et al., 2006; Wilson et al., 2012). Nevertheless, it is transmissible to cattle, sheep, goats, ferrets, hamsters, bank voles, minks, raccoons, and squirrel monkeys when intracerebrally inoculated (Sigurdson, 2008). Recent studies have provided evidence of the capacity *in vitro* of this CWD to convert human $PrP^C$ after either passage in Tg mice expressing cervid $PrP^C$ or serial PMCA amplification in deer $PrP^C$ substrate (Barria et al., 2011). In addition, CWD has been successfully transmitted to squirrel monkeys, albeit less efficiently than the epidemic BSE (Marsh et al., 2005; Race et al., 2009b). Despite these findings, there is no epidemiological evidence to date for a zoonotic role of the CWD agent.

CWD surveillance and control measures have been implemented across the USA and Canada to reduce disease spread, mitigate the economic losses due to less recreational hunting, and control its potential cross-species transmission (Williams, 2005; Joly et al., 2009; Wasserberg et al., 2009). Challenges associated with CWD include i) the high prion contamination of the environment due to the excretion of the agent by multiple vias and the high persistence of its infectivity (Saunders et al., 2008; Saunders et al., 2011; Smith et al., 2011), ii) the geographical spread of the agent as a result of the natural migration of cervids and scavengers, and iii) the existence of different CWD strains (Angers et al., 2010).

# IV. OBJECTIVES

**The general objective of this thesis is to improve our understanding of the molecular mechanisms underlying susceptibility/resistance to prion infection.** To address this task a number of specific aims were pursued:

1. To study the individual role of the polymorphic variants found naturally in goat and sheep *prnp* in the susceptibility/resistance to scrapie and BSE infection in transgenic mice.

   1.1 To generate a panel of transgenic mouse lines each expressing either the goat wild type *prnp* or one goat ($M_{142}$, $H_{154}$, $Q_{211}$ and $K_{222}$) or sheep ($R_{171}$ and $K_{176}$) polymorphic variant of this wild type *prnp.*

   1.2. To determine the suitability of the transgenic mouse line expressing the goat/sheep wild type *prnp* for analyzing the susceptibility of goats and sheep to different prion strains.

   1.3. To compare individual susceptibility to different prion strains in the different mouse lines expressing each polymorphic variant with the mouse line expressing the wt-$PrP^C$.

   1.4. To analyze the protective effect of the *prnp* polymorphic variants associated with prion resistance over the prion replication of the wild type allele.

2. To confirm in a natural host (goats) the reliability of the results from mouse models:

   2.1. To analyze the effect of *prnp* polymorphic variants on the susceptibility of goats to oral and intracerebral infection with classical scrapie.

   2.2. To evaluate the effect of *prnp* polymorphic variants on the susceptibility of goats to oral infection with goat-BSE.

The experimental design uses two complimentary models, Tg mice and goats. The Tg model permitted us to analyze from a molecular point of view the role of polymorphic variants of the prion protein in infection with scrapie and BSE. Transmission studies in goats – the natural host for scrapie and, potentially, for BSE – allowed us to evaluate the ability of our Tg models to predict prion infection in goats.

# V. MATERIALS AND METHODS

## 1. Studies in goat and sheep PrP transgenic mice

### 1.1 Mouse model

#### *1.1.1 Generation of transgenic mouse lines*

##### *1.1.1.1 Plasmid construction*

A schematic representation of the generation of all Tg mouse lines is given in Fig. 9. Transgenic mouse lines expressing either the goat wild type-$PrP^C$ or the $K_{222}$-$PrP^C$ variant were previously generated by our group. Briefly, the Open Reading Frame (ORF) of goat wild type (wt) PrP gene (*prnp*) (GenBank accession number AF117316.1) was isolated by PCR amplification and subcloned into the pGEM-T Easy vector system (Promega) (Fig. 9). The obtained goat wt *prnp* ORF was excised from the cloning vector and inserted into the expression vector MoPrP.Xho (Borchelt et al., 1996), resulting in the plasmid pMo-**Go**PrP.Xho. This vector contains the murine PrP (muPrP) promoter, exon 1, intron 1, exon 2, and 3'-untranslated sequences flanked by two *XhoI* restriction sites but could be distinguished from the wt murine PrP gene because of the absence of intron 2. pMo-**Go**PrP.Xho plasmid was directedly mutated to generate a $K_{222}$-PrP plasmid (pMo-Go**K222**-PrP.Xho) by using a QuikChange II XL kit (Stratagene, CA) with specific oligonucleotides (5´-GTGCATCACCCAGTACAAGAGAGAATCCCAGGC-3´ and 3´-GCCTGGGATTCTCTCTTGTACTGGGTGATGCAC-5´), following the manufacturer's instructions.

$M_{142}$, $H_{154}$, $R_{171}$, $K_{176,}$ and $Q_{211}$-PrP plasmids were new-generated for the present study by directed mutation of the plasmid pMo-**Go**PrP.Xho, as previously carried out for the $K_{222}$-PrP plasmid. Mutations consisting of single nucleotide substitutions were obtained using the same QuikChange II XL kit (Stratagene, CA) with other specific oligonucleotides (Table 1).

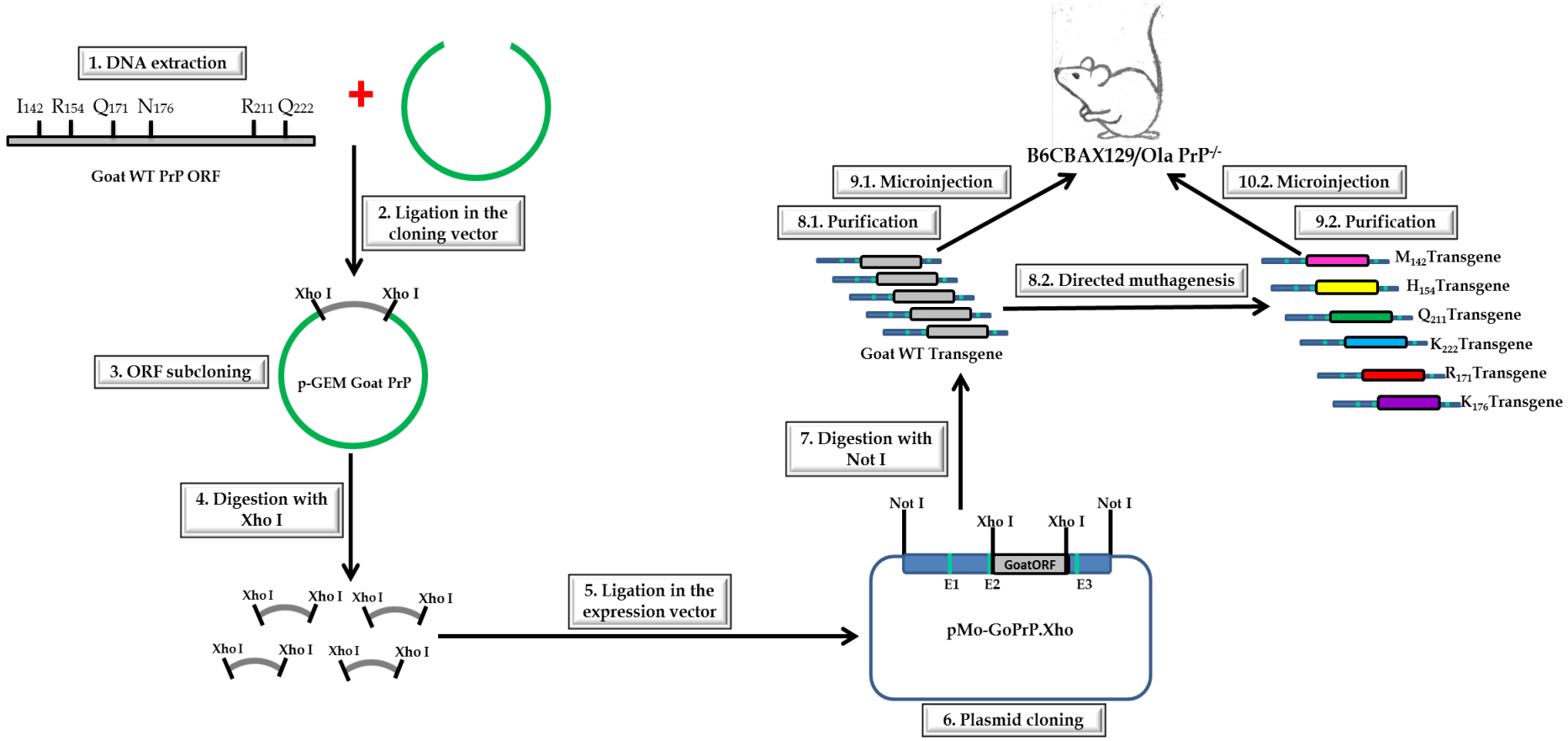


**Figure 9. Schematic representation of the generation of the goat and sheep PrP transgenic mice.**

**Table 3.** Oligonucleotides used for generating the different $PrP^C$ variants-plasmids

| $PrP^C$ variant | Nucleotide substitution | aa exchange | Oligonucleotide used for directed mutagenesis | $PrP^C$ variant-plasmids |
|---|---|---|---|---|
| **K222** | CAG to **A**AG | Q to K | Forward: 5′-GTGCATCACCCAGTAC **A**AGAGAGAATCCCAGGC-3′ Reverse: 5′- GCCTGGGATTCTCTCT **T**GTACTGGGTGATGCAC-3 | pMo-Go**K222**-PrP.Xho |
| **M142** | ATA to AT**G** | I to M | Forward: 5′-GCCATGAGCAGGCCTC TTAT**G**CATTTTGGCAATGAC -3′ Reverse: 5′-GTCATTGCCAAAATG**C** ATAAGAGGCCTGCTCATGGC -3′ | pMo-Go**M142**-PrP.Xho |
| **H154** | CGT to C**A**T | R to H | Forward: 5′-CTATGAGGACCGTTAC TATCATGAAAAC**A**TGTACCGTT ACC -3′ Reverse: 5′-GGTAACGGTACA**T**GTT TTCATGATAGTAACGGTCCTCAT AG - 3′ | pMo-Go**H154**-PrP.Xho |
| **R171** | CAG to CG**G** | Q to R | Forward: 5′-CTACAGACCAGTGGAT CG**G**TATAGTAACCAGAACAAC-3′ Reverse: 5′-GTTGTTCTGGTTACTAT A**C**CGATCCACTGGTCTGTAG-3′ | pMo-Go**R171**-PrP.Xho |
| **K176** | AAC to AA**A** | N to K | Forward: 5′-GTGGATCAGTATAGTA ACCAGAA**A**AACTTTGTGCATGA CTGTGTC-3′ Reverse: 5′-GACACAGTCATGCACA AAGTT**T**TTCTGGTTACTATACTG ATCCAC-3′ | pMo-Go**K176**-PrP.Xho |
| **Q211** | CGA to C**A**A | R to Q | Forward: 5′-CATCAAGATAATGGAG C**A**AGTGGTGGAGCAAATGTGC-3′ Reverse: 5′-GCACATTTGCTCCACCA C**T**TGCTCCATTATCTTGATG-3′ | pMo-Go**Q211**-PrP.Xho |

### *1.1.1.2 Microinjection of DNA constructions*

All different transgenes ($M_{142}$, H154, $R_{171}$, $K_{176}$, and $Q_{211}$) were excised from their respective expression vectors (pMo-Go**M142**-PrP.Xho, pMo-Go**H154**-PrP.Xho,

pMo-Go**R171**-PrP.Xho, pMo-Go**K176**-PrP.Xho, and pMo-Go**Q211**-PrP.Xho) using the restriction endonuclease NotI, which gave DNA fragments of approximately 12 kb corresponding to the murine promoter and the goat PrP ORF (Fig. 9).

The transgenes of each variant were recovered from a 0.7% agarose gel (Pronadisa) using a DNA purification kit (Elu-Quik; Schleicher & Schuell). Then, DNAs were dissolved in TE (10mM Tris, pH 7.4, 0.1mM EDTA) at a final concentration of 2–6 ng/ml. Finally, DNAs were microinjected into pronuclear-stage embryos collected from superovulated B6CBAF1 females mated with 129/Ola males carrying a null mutation in their endogenous *prnp* (Manson et al., 1994). For each transgene, multiple embryos were microinjected at the Centro Nacional de Biotecnología (CNB-CSIC) and different number of founders was obtained (see Results, Table 7).

#### *1.1.1.3 Screening of founders*

DNAs were extracted from founders' tail biopsy specimens using an Extract-N-Amp tissue PCR kit (Sigma-Aldrich) following the manufacturer's instructions. The presence of the goat or sheep transgene in these founders was identified by PCR amplification using specific primers for mouse PrP exon 2 and the goat PrP ORF. The primers used were 5'-CATTCTGCCTTCCTAGTGGTACC-3' and 5'-GCTTGTTCCACTGACTGTGGC-3'. muPrP$^{+/-}$ goPrP$^{+/-}$ founders were backcrossed with homozygous PrP null animals (muPrP$^{-/-}$) to obtain mice homozygosis for the null mutation (muPrP$^{-/-}$ goPrP$^{+/-}$). The absence of the murine PrP ORF in the generated transgenic mice was confirmed by PCR amplification using the primers 5'-ATGGCGAACCTTGGCTACTGGC-3' and 5'-GATTATGGGTACCCCCTCCTTGG-3'.

#### *1.1.1.4 Analysis of transgene transmission to the offspring*

The offspring obtained from breeding each founder (muPrP$^{+/-}$ goPrP$^{+/-}$) with *prnp* null mice (*prnp* mu-/- goPrP$^{-/-}$) (Manson et al., 1994) was studied for the presence of the goat or sheep transgene by PCR as described above. Transgene

transmission to ≤50% of the progeny (following Mendel´s principle of segregation) indicated that only one set of transgene copy was inserted in the mice genome (Fig. 10). Founders with more than one integration site were discarded.

### *1.1.1.5 Analysis of brain $PrP^C$ expression in hemizygous transgenic mouse lines*

Whole brains from mice, goats, and sheep were homogenized in extraction buffer (1% (w/v) NP40, 1% (w/v) sodium deoxycholate, and 10mM EDTA in PBS pH 7.4 + complete™ cocktail of protease inhibitors (Roche). Samples were precleared by centrifugation at 2,000 X *g* for 5 min, after which an equal volume of 2X SDS reducing sample loading buffer was added to all samples, and each was boiled for 5 min. 20 µl of each sample were loaded into a 12% Criterion XT Bis-Tris Gel (BioRad). Electrophoresis was performed in XT MES running buffer (BioRad) at 150V for 80 min. Proteins were electrophoretically transferred to PVDF membranes (Millipore) at 400 mA for 90 min. Membranes were blocked O/N with 2% (w/v) Bovine Serum Albumin (BSA) fraction V (Sigma-Aldrich) in PBS-Tween (BSA-PBST).

For the immunoblotting experiments, membranes were incubated for an hour with monoclonal antibodies (mAbs) FH11 (Foster et al., 1996b) or 12B2 (Yull et al., 2006) at a final concentration of 1µg/ml in BSA-PBST. FH11 recognizes the goat PrP amino-terminal region (amino acids 23–85), while 12B2 recognizes the $_{93}$WGQGG$_{97}$ epitope of the goat PrP sequence. Membranes were washed three times for 5 min with PBST to minimize background and remove unbound Ab. Immunocomplexes were detected with horseradish peroxidase-conjugated anti-mouse IgG (Amersham GE Life Sciences) at a final concentration of 1µg/ml in BSA-PBST after incubating the membranes for an hour. Membranes were washed again three times for 5 min with PBST, and immunoreactivity was visualized using chemiluminescence with Pierce® ECL 2 (Thermo Scientific) in a Gel imaging system (Bio-Rad ChemiDoc™ XRS). The signal was recorded during 15 min of exposure time.

### *1.1.1.6 Analysis of animal health and physiological behavior*

All the founders, as well as 6–7 animals from each progeny, were housed until the end of their lifespans and monitored for the presence of any signs of neurological or other physiological alterations. Their brains were histopathologically analyzed to discard the possibility of neurological alterations.

### *1.1.1.7 Obtaining homozygous goat and sheep $PrP^C$ transgenic mice*

Homozygosity for each goat or sheep PrP transgene (WT, $K_{222}$, $M_{142}$, $H_{154}$, $R_{171}$, $K_{176}$, and $Q_{211}$) was obtained by interbreeding hemizygous goat or sheep PrP mice ($muPrP^{-/-}$ $goPrP^{+/-}$) from the same transgenic mouse line rendering $muPrP^{-/-}$ $goPrP^{+/-}$ (50%), $muPrP^{-/-}$ $goPrP^{+/+}$ (25%), and $muPrP^{-/-}$ $goPrP^{-/-}$ (25%) (Fig. 10). Homozygosity was confirmed by backcrossing potential homozygous transgenic mice ($muPrP^{-/-}$ $goPrP^{+/+}$) with homozygous PrP null animals ($muPrP^{-/-}$) and rendering 100% of hemizygous ($muPrP^{-/-}$ $goPrP^{+/-}$) animals. $PrP^C$ expression in the brain of homozygous mice was analyzed using Western blot as for hemizygous mice (see section 1.1.1.5). The brains of homozygous mice should express twice as much $PrP^C$ as those of their hemizygous counterparts.

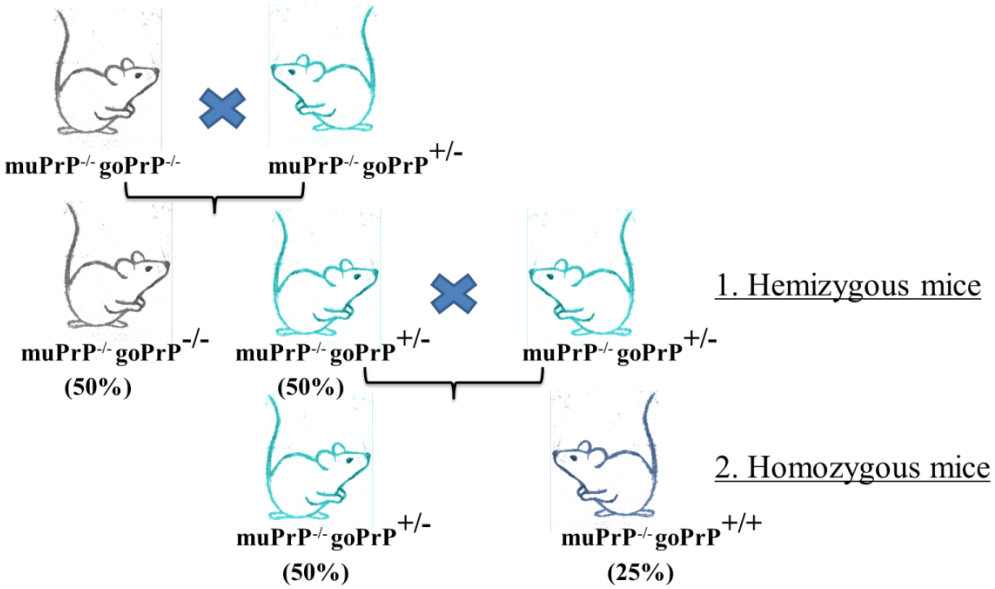


**Figure 10. Hemizygous and homozygous transgenic mice generation.** Schematic representation of the generation of hemizygous and homozygous mice for each different goat and sheep PrP transgenic mouse lines.

#### *1.1.1.8 Obtaining heterozygous Q/K$_{222}$ transgenic mice*

Heterozygous Q/K$_{222}$ transgenic mice were obtained by mating homozygous K$_{222}$-Tg516 (muPrP$^{-/-}$ goK$_{222}$PrP$^{+/+}$) and homozygous Wt-Tg501 (muPrP$^{-/-}$ goQ$_{222}$PrP$^{+/+}$) mice, which rendered 100% Q/K$_{222}$ (muPrP$^{-/-}$ goQ$_{222}$PrP$^{+/-}$ goK$_{222}$PrP$^{+/-}$).

### 1.2 Transmission studies in mice

#### *1.2.1 TSE isolates collection and characterization*

Transgenic mice expressing different genotypes of goat and sheep *prnp* were challenged with a wide variety of TSE agents, including both classical and atypical scrapie isolates, as well as BSE isolates (see Tables 2–5 for isolates information). Inocula were prepared from infected brains as 10% (weight/volume) homogenates in 5% glucose. Brain homogenates from PrP$^{res}$ positive mice were pooled for further passaging. When all mice were scored negative for PrP$^{res}$ on the primary passage, PrP$^{res}$-negative brain homogenates were used for the second passage.

#### *1.2.1.1. Molecular characterization (genotyping)*

The *prnp* ORF was amplified from the purified genomic DNA in the brain homogenate of each isolate by PCR using specific oligonucleotides (5'-CTCGAGATCATGGTGAAAAGCCACATAGGC-3' and 3'-CTCGAGCTATCCTACTATGAGAAAAATGAG-5'). Then, the *prnp* ORF was sequenced by the department of Sequencing Services of the Centro de Investigación en Sanidad Animal (CISA, INIA). The polymorphic variants that each isolate harbors are indicated in tables 2–5, "PrP$^{Sc}$ genotype".

**Table 4.** Description of the goat classical scrapie isolates used in this work

| Isolate | Origin (local code) | $PrP^{Sc}$ genotype | Description and references | Supplier |
|---|---|---|---|---|
| **Goat-Sc F2** | Fr (CP40) | wt[a]; $S/P_{240}$ | Classical scrapie from an experimentally infected goat | INRAN[1] |
| **Goat-Sc F3** | Fr (CDP1028) | wt; $P/P_{240}$ | Classical scrapie from a naturally infected goat | INRAN |
| **Goat-Sc F6** | Fr (CP2119) | wt; $S/P_{240}$ | Classical scrapie from a naturally infected goat | INRAN |
| **Goat-Sc F10** | Fr (2143) | wt; $S/P_{240}$ | Classical scrapie from a naturally infected goat | INRAN |
| **Goat-ScF11** | Fr (2154) | wt; $I/M_{142}$; $P/P_{240}$ | Classical scrapie from an experimentally infected goat | INRAN |
| **Goat-Sc F14** | Fr (9041) | wt; $I/M_{142}$; $S/P_{240}$ | Classical scrapie from a naturally infected goat | INRAN |
| **Goat-ScF16** | Fr (9135) | wt; $I/M_{142}$; $S/P_{240}$ | Classical scrapie from a naturally infected goat | INRAN |
| **Goat-Sc I2** | It (114921/1/1) | wt; $S/S_{240}$ | Classical scrapie from a naturally infected goat | IZSTO[2] |
| **Goat-Sc I3** | It (121429/1/1) | wt; $P/P_{240}$ | Classical scrapie from a naturally infected goat | IZSTO |
| **Goat-Sc I4** | It (128710/1/1) | wt; $R/Q_{211}$; $S/P_{240}$ | Classical scrapie from a naturally infected goat | IZSTO |
| **Goat-Sc I9** | It (85792/1/1) | wt; $R/H_{143}$; $S/P_{240}$ | Classical scrapie from a naturally infected goat | IZSTO |
| **Goat-Sc I12** | It (144508/1/1) | wt; $S/P_{240}$ | Classical scrapie from a naturally infected goat | IZSTO |
| **Goat-Sc G2** | Gr (1883) | wt; $P/P_{240}$ | Classical scrapie from a naturally infected goat | CERTH[3] |
| **Goat-Sc G3** | Gr (1878) | wt; $R/H_{143}$; $P/P_{240}$ | Classical scrapie from a naturally infected goat | CERTH[3] |
| **Goat-Sc N1** | Ne (1) | wt; $R/H_{143}$; $S/P_{240}$ | Classical scrapie from a naturally infected goat | CIDC[4] |
| **Goat-Sc N2** | Ne (2) | wt; $R/H_{143}$; $S/P_{240}$ | Classical scrapie from a naturally infected goat | CIDC |
| **Goat-Sc N3** | Ne (3) | wt; $P/P_{240}$ | Classical scrapie from a naturally infected goat | CIDC |
| **Goat-Sc S2** | Sp (C-163) | wt; $S/P_{240}$ | Classical scrapie from a naturally infected goat | UNIZAR[5] |
| **Goat-Sc S3** | Sp (C-645) | wt; $P/P_{240}$ | Classical scrapie from a naturally infected goat | UNIZAR |
| **Goat-Sc UKA2** | UKA2 | wt; $S/P_{240}$ | Classical scrapie from a naturally infected goat | UEDIN[6] |
| **Goat-Sc UKB2** | UKB2 | wt; $G/S_{127}$; $P/P_{240}$ | Classical scrapie from a naturally infected goat | UEDIN[6] |
| **Goat-Sc UKD2** | UK D2 | wt; $G/S_{127}$; $P/P_{240}$ | Classical scrapie from a naturally infected goat | UEDIN |
| **Goat-Sc ZYP13** | C1 | wt; $P/P_{240}$ | Classical scrapie from a naturally infected goat | TVCN[7] |
| **Goat-Sc ZYP21** | C2 | wt; $P/P_{240}$ | Classical scrapie from a naturally infected goat | TVCN |
| **Healthy goat brain** | - | wt; $P/P_{240}$ | Brain from a non-infected goat | INRAN |

[a] Wild type (wt) goat prion protein genotype: $A_{136}R_{154}Q_{171}$/ $A_{136}R_{154}Q_{171}$. // [1] French National Institute for Agricultural Research (INRA), Nouzilly, France. [2] Istituto Zooprofilattico Sperimentale del Piemont, Italy. // [3] CERTH-INA, Thessaloniki, Greece. // [4] Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands. // [5] Universidad de Zaragoza, Spain. // [6] Roslin Institute, University of Edinburgh (UEDIN-Roslin), Edinburgh, UK. // [7] Toumazos Veterinary Center, Nicosia, Cyprus.

**Table 5.** Description of the sheep classical scrapie isolates used in this work

| Isolate | Origin (local code) | $PrP^{Sc}$ genotype | Description and references | Supplier |
|---|---|---|---|---|
| **Sheep-Sc21** | Fr (CH0064) | wt[a]; $S/S_{240}$ | Classical scrapie from a naturally infected sheep | $INRA_N$[1] |
| **Sheep-Sc Langlade** | PD377 | wt; $S/S_{240}$ | Classical scrapie from a naturally infected sheep | $INRA_T$[2] |
| **Sheep-Sc 09** | - | wt; $F/F_{141};S/S_{240}$ | Pool of terminally ill mice TgOV IX infected with one classical scrapie isolate | FLI[3] |

[a] Wild type (wt) goat prion protein genotype: $A_{136}R_{154}Q_{171}/ A_{136}R_{154}Q_{171}$. // [1] French National Institute for Agricultural Research (INRA), Nouzilly, France.

[2] French National Institute for Agricultural Research (INRA), Toulouse, France. // [3] Friedrich-Loeffler-Institut, InselRiems, Germany.

**Table 6.** Description of the atypical isolates used in this work

| Isolate | Origin (local code) | $PrP^{Sc}$ genotype | Description and references | Supplier |
|---|---|---|---|---|
| **Goat-Sc I15** | It (87016/1/1) | wt[a]; $H/R_{154}$ $S/P_{240}$ | Atypical scrapie from a naturally infected goat | IZSTO[1] |
| **Sheep-Sc M45** | - | wt; $S/S_{240}$ | Atypical scrapie from a naturally infected sheep | NEIKER[2] |
| **Sheep-Sc engavagen** | Sc3080 | wt; $F/F_{141};S/S_{240}$ | Atypical scrapie from a naturally infected sheep | NVT[3] |
| **Sheep-Sc TOA4** | TOA4 | wt; $H/Q_{171};S/S_{240}$ | Atypical scrapie from a naturally infected sheep | CReSA[4] |
| **Sheep-Sc Nor98/Tg338** | - | wt;$V/V_{136};S/S_{240}$ | Pool of cerebellum from terminally ill mice Tg338 infected with an atypical scrapie isolate (Laude et al., 2002) | NVT |

[a] Wild type (wt) goat prion protein genotype: $A_{136}R_{154}Q_{171}/ A_{136}R_{154}Q_{171}$. // [1] Istituto Zooprofilattico Sperimentale del Piemont, Italy. // [2] Instituto Vasco de Investigación y Desarrollo Agrario (NEIKER), Vizcaya, Spain. // [3] Norwegian Veterinary Institute, Oslo, Norway. // [4] Centre de Recerca en Sanitat Animal (CReSA-PRIOCAT), Barcelona, Spain.

**Table 7.** Description of the BSE isolates used in this work

| Isolate | Origin (local code) | $PrP^{Sc}$ genotype | Description and references | Supplier |
|---|---|---|---|---|
| **Goat-BSE1** | Fr (CH0064) | wt[a]; $S/S_{240}$ | Terminally ill goat intracerebrally inoculated with Ca-BSE isolate | $INRA_N$[1] |
| **Goat-BSE2** | Fr (CH1075) | wt; $S/P_{240}$ | Terminally ill goat intracerebrally inoculated with Ca-BSE isolate | $INRA_N$ |
| **Sheep-BSE** | Fr ($ARQ_0$) | wt;$S/S_{240}$ | Pool of terminally ill ARQ/ARQ sheep inoculated with Ca-BSE isolate cow (Espinosa et al., 2007a) | $INRA_N$ |
| **Cattle-BSE** | Fr (139) | Cattle wt | BSE from a naturally infected cow (Espinosa et al., 2007a) | $INRA_N$ |
| **Cattle-BSE L** | 02.2528 | Cattle wt | BSE from a naturally infected cow | $INRA_N$ |

[a] Wild type (wt) goat prion protein genotype: $A_{136}R_{154}Q_{171}/ A_{136}R_{154}Q_{171}$. // [1] French National Institute for Agricultural Research (INRA), Nouzilly, France.

### *1.2.1.2. $PrP^{res}$ biochemical characterization*

$PrP^{res}$ was analyzed for each isolate using Western blotting (WB), as previously described (Padilla et al., 2011). Briefly, 100 µl of the isolate (10% brain homogenate) was diluted in a 10% (w/v) negative sheep brain homogenate to obtain a 200 µl final volume. Homogenates were incubated for 15 min at 37ºC with 200 µl of a 2% proteinase K solution (in buffer A). $PrP^{res}$ was recovered as a pellet following the addition of 200 µl of buffer B (Bio-Rad) and a centrifugation at 15,000 x *g* for 7 min at 20ºC. Supernatants were discarded and pellets were dried over absorbent paper for 5 min. Pellets were solubilized in 50 µl of Laemmli buffer and samples were incubated for 5 min at room temperature, solubilized, and heated at 100ºC for 5 min. Samples were centrifuged at 20,000 x *g* for 15 min at 20ºC and supernatants were recovered and loaded into a 12% Criterion XT Bis-Tris Gel (BioRad). Electrophoresis was performed in XT MES running buffer (BioRad) at 150V for 80 min. Proteins were electrophoretically transferred to PVDF membranes (Millipore) at 400 mA for 90 min. Membranes were blocked O/N with BSA-PBST.

For immunoblotting, membranes were incubated for an hour with Sha31 mAb (Feraudet et al., 2005), which recognizes the $_{148}$YEDRYYRE$_{155}$ epitope of the goat PrP sequence, at 1µg/ml dilution in BSA-PBST. Membranes were washed three times for 5 min with PBST. Immunocomplexes were detected with horseradish peroxidase-conjugated anti-mouse IgG (Amersham GE Life Sciences) at 1µg/ml dilution in BSA-PBST after incubating the membranes for an hour. Membranes were washed again three times for 5 min with PBST and immunoreactivity was visualized by chemiluminescence with Pierce® ECL 2 (Thermo Scientific) in a Gel imaging system (Bio-Rad ChemiDoc™ XRS). Signal was recorded during 15 min of exposure time.

For detecting atypical scrapie $PrP^{res}$, the same WB procedure was followed (Padilla et al., 2011), although the membranes were incubated with 9A2 mAb (Yull et al., 2006), which recognizes the $_{102}$WNK$_{104}$ epitope of the goat PrP

sequence, at 1µg/ml dilution in BSA-PBST for immunoblotting, and the signal was recorded for 30 min of exposure time.

### *1.2.2 Mice inoculation*

Groups of 6–9 individually identified animals (6–7-weeks old) were anesthetized with isoflurane (Isoba vet Schering-Plough S.A.) and were intracerebrally inoculated using a 25-gauge disposable hypodermic needle with 20 µl of 10% brain homogenate in the right parietal lobe. As a control, 6 or 7 animals of each line were inoculated with healthy goat brain to rule out the possibility of spontaneous prion disease developing.

### *1.2.3 Clinical status monitoring*

After inoculation, mice were observed daily and their neurological status assessed twice a week. When the progression of the disease was evident or at the end of their life span (≈650 days), mice were humanely euthanized by cervical dislocation. A mouse was considered positive for neurological disease when it displayed two or three out of the previously described 10 signs of neurological dysfunction (Scott et al., 1989; Scott et al., 1993b). Definitive diagnoses were made when one confirmatory sign of prion disease (ataxia, generalized tremor, loss of righting reflex, limb paralysis, extensive pilo-erection, or sustained hunched posture) was observed, at which point the animals were culled.

### *1.2.4 Euthanasia and tissue sampling*

During necropsy, part of each brain was immediately fixed in neutral-buffered 10% formalin (4% 2-formaldehyde) for determining PK resistant PrP accumulations using immunohistochemistry (IHC) and/or Paraffin-embedded Tissue (PET) blot and spongiform degeneration by histopathology. The other part was homogenized as 10% (w/v) in sterile 0.9 NaCl and harvested at -20$^{o}$C for detecting the presence of $PrP^{res}$ by Western blot (WB) and/or further passaging. Survival time (ST) was expressed as the mean number of survival

days postinoculation (dpi) for all the $PrP^{res}$-positive mice, with the standard error of the mena (SEM) included. Attack rate (AR) was determined as the proportion of $PrP^{res}$-positive mice among all the mice inoculated.

### *1.2.5 Brain $PrP^{res}$ detection by Western blot*

A total of 175 mg of whole brain tissue was homogenized in 5% glucose in distilled water in grinding tubes (Bio-Rad) and adjusted to 10% (w/v) by using a TeSeETM Precess 48™ homogenizer (Bio-Rad) following the manufacturer's instructions. Homogenates were pressed through 0.4 mm needles of a calibration syringe and immediately frozen at -20ºC. The presence of $PrP^{res}$ in transgenic mouse brains was determined by Western blotting following the same procedure as in section 1.2.1.2. In all, 100 µl of 10% brain homogenate were analyzed in each WB experiment. For immunoblotting, membranes were incubated with Sha31 and immunocomplexes were detected with horseradish peroxidase-conjugated anti-mouse IgG (Amersham GE Life Sciences). For detecting atypical scrapie $PrP^{res}$, the same WB procedure was followed, although the membranes were incubated with 9A2 mAb and the signal was recorded for 30 min of exposure time.

### *1.2.6 Histopathological analysis*

All procedures involved in the histopathological analysis of infected mouse brains were performed in the laboratory of Dr. Olivier Andréoletti at the École Nationale Vétérinaire in Toulouse, France.

Brain samples were immediately fixed in neutral-buffered 10% formalin (4% 2-formaldehyde) during necropsy. Later, samples were dehydrated in a HMP 110 processor for 16 hours, paraffin-embedded in cassettes using a Tissue-Tek® TEC III Embedding Center System and harvested at -20ºC.

#### *1.2.6.1 Hematoxylin-Eosin staining*

Paraffin-embedded (paraplast plus Kendall) brain samples were cut into 3µm thick sections using a microtome and collected onto adhesive-treated slides

(adhesive-treated ChemMate Capillary Gap microscope slides (DAKO)). Slides were dried overnight at 37ºC and then deparaffinated and rehydrated. Finally, brain samples were stained with hematoxylin (Mayer´s hemalum solution: RAL 320550-2500/320554) and eosin (HE) and dried before microscopic analysis. Lesion profiles in the different coronal sections of the brain samples were established following the standard method described by Fraser and Dickinson (Fraser and Dickinson, 1968). This method scores the degree of vacuolation in the range 0–4 in nine gray and three white areas of the brain: dorsal medulla (G1), cerebellar cortex (G2), superior colliculus (G3), hypothalamus (G4), medial thalamus (G5), hippocampus (G6), septum (G7), medial cerebral cortex at the level of the thalamus (G8), and at the level of the septum (G9), cerebellum (W1), mesencephalic tegmentum (W2), and pyramidal tract (W3).

#### *1.2.6.2 Paraffin-embedded Tissue blot*

Paraffin-embedded brain samples were cut 3µm thick sections using a Leica® RM 2135 microtome and collected onto 0.45 µm nitrocellulose membranes for Paraffin-embedded Tissue (PET) blotting. Membranes were dried O/N at 56ºC before being deparaffinated and rehydrated. For PK digestion, membranes were incubated with PK 250 µg/ml (PK recombinant, PCR Grade, Roche) for two hours at 55°C. Then, membranes were denaturated in guanidium isothiocyanate 3 M solution for 10 min at room temperature and blocked with 0.2% BSA (Sigma Aldrich) in PBST dilution. For immunodetection, membranes were incubated with Sha-31 mAb (4 µg/ml saturation buffer) for an hour at room temperature. Immunocomplexes were detected with an alkaline phosphatase-coupled secondary Ab (Polyclonal rabbit antimouse Inmunoglobuline, Dako) at 2µl/ml dilution in a saturation buffer. Immunoreactivity was revealed using NBT/BCIP substrate chromogen (Sigma B-5655).

## 2. Goat models

All procedures involving the goat transmission studies (goat mating, sequencing and inoculation, clinical status monitoring, euthanasia, tissue sampling, and histopathological analysis) were performed either in the laboratory of Dr. Olivier Andréoletti at the École Nationale Vétérinaire in Toulouse (France) or in the laboratory of Dr. Martin Groshup at the Institute for Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut (FLI), Insel Riems (Germany) in the framework of the StrepBSE in goat EU project (UE-FP6-2005-FOOD4B-036353). These procedures are described below and will help in the understanding of the experiments.

### 2.1 Goat mating and sequencing

The goats kids used in the experiment were produced by direct mating of *prnp*-sequenced Alpine and Saanen female goats and bucks. Parents were selected from three herds managed by the French National Agronomic Institute (INRA). Selection was based on the *prnp* polymorphisms at codons 142 (I/M), 154 (R/H), 211 (R/Q), and 222 (Q/K). Animals were then naturally mated to produce the goats used in the experimental inoculation.

The Exon3 of the *prnp* of each goat kid was sequenced as previously described (Arsac et al., 2007). Briefly, DNA was directly recovered from the brain stem (30 mg) using a commercial DNA extraction kit (Qiaprep DNeasy Minikit (QIAGEN, Courtaboeuf, France) following the manufacturer's recommendations. The complete ORF sequence of the *prnp* was determined using several oligonucleotides: 3´-GTGGGCATTTGATGCTGACAC-5´; 5´-TGGTTGGGGTAACGGTACATG-3´; 5´-TCAGCCCCATGGTGGTGGCT-3´ and 3´-CTGCAGGTAGACACTCCCTCC-5´.

## *2.2 Transmission studies in goats*

### *2.2.1 Goat inoculation*

#### *Goat bioassay with scrapie at INRA*

Within 48 hours of birth a group of goat kids received 1.5g of brain-equivalent material through natural suckling (1% diluted stock inoculum in glucose 5%). A second inoculation (same material and route) was performed at the age of 30 days. In parallel, a second group of goat kids were housed until they were six months of age. At this age, all the goats were anesthetised (Ketamine/Valium) and 400µL of the stock inoculum was injected into their temporal cortex. The inoculum was derived from a single natural field scrapie case (clinical) obtained from a 3.5-year-old goat with the wt *prnp* genotype.

#### *Goat bioassay with BSE at FLI*

At 6–7 months of age goats harboring WT ($R_{211}Q_{222}$/RQ), R/$Q_{211}$, or Q/$K_{222}$ genotypes received a total dose of approximately 1g brain-equivalent material from BSE first passaged in goat. This goat BSE material was obtained by pooling brainstems from three WT goats experimentally inoculated with a mixture of four cattle BSE field cases (Foster et al., 1993; Goldmann et al., 1996). The inoculum was orally administered at 1:5 (w/v) dilution in sterile 0.9 NaCl; each goat received 5ml of pooled goat brain homogenate.

### *2.2.2 Clinical status monitoring, euthanasia, and tissue sampling*

Goats were regularly monitored for the presence of clinical signs, i.e. cachexia, alopecia, lethargy, and abnormalities in sensations and movements. Goats were euthanatized at the clinical stage or at scheduled times (Tables 6 and 7) using Ketaminhydrochlorid (10-15 mg/kg) and Xylazinhydrochlorid for anesthesia followed by euthanasia with T61 (4-6ml/50 kg body weight). During necropsy, a set of tissues including brain, lymph node popliteal is, psoas major, and retractor bulbi muscles was collected under TSE sterile conditions and harvested at -80°C.

### *2.2.3 Histological analysis*

A part of each sample was immediately fixed in neutral-buffered 10% formalin (4% formaldehyde). Subsequently, these samples were treated for one hour with 98% formic acid and rinsed in tap water for 40 min before dehydration and embedding in paraffin. After rehydration, 3-µm-thick brain slices were stained with hematoxylin and eosin (HE) and a histopathological examination was carried out. For detecting $PrP^{Sc}$ deposits, immunohistochemistry (IHC) analyses were performed as previously described (Kaatz et al., 2012), using 6C2 (Central Veterinary Institute of Wageningen UR, Lelystad, Netherlands) and F99 (VMRD, Pullman, USA) monoclonal antibodies (mAb).

Subsequently, the sections were pretreated by incubation with 98% formic acid (15 min), followed by autoclaving in citrate buffer for 20 min at 121°C. The endogenous peroxidase was inhibited using 3% $H_2O_2$ in methanol for 30 min. The primary antibodies were diluted in goat serum: the 6C2 at 1:50 to 1:150 and the F99 at 1:4000, and then incubated for two hours at room temperature. Negative control sections were treated only with goat serum. As a secondary Ab the EnVision™ reagent (Dako, Hamburg, Germany) containing a peroxidase-conjugated polymer backbone was used. The incubation time for these sections was 30 min at room temperature. The slides were finally developed in diaminobenzidine tetrahydrochloride (Fluka, Steinheim, Germany) and counterstained with Mayer's haematoxylin. All sections were examined by light microscopy.

### *2.2.4 $PrP^{res}$ detection in different goat tissues by Western blot*

$PrP^{res}$ was detected in tissues from goat BSE orally inoculated goats by WB as mentioned above (see section 1.2.1.2). In all, 100 µl of each tissue 10% (w/v) homogenate were analyzed. For immunoblotting, membranes were incubated with Sha31 mAb and immunocomplexes were detected with horseradish peroxidase-conjugated anti-mouse IgG (Amersham GE Life Sciences).

## 2.3 Prion infectivity in goat tissues

### *2.3.1 Prion infectivity in Goat BSE inoculum*

Wt-Tg501 and BoPrP-Tg110 mouse lines were used for titration of the Goat BSE isolate. The BoPrP-Tg110 mouse line was established and has been described elsewhere (Castilla et al., 2003). BoPrP-Tg110 transgenic mice express bovine PrP protein under the murine PrP promoter in a murine PrP null ($muPrP^{-/-}$) background. $PrP^{C}$ expression levels in this mouse line are approximately 8-fold higher than the $PrP^{C}$ levels found in cow brain homogenates.

Groups of 6–9 individually identified Wt-Tg501 and BoPrP-Tg110 (6–7-weeks old) mice were intracerebrally inoculated with 10-fold serial dilution of the Goat-BSE isolate (Foster et al., 1993; Goldmann et al., 1996). Mice inoculation, clinical status monitoring, euthanasia, tissue sampling, and brain $PrP^{res}$ detection by Western blot were performed as previously described (see sections 1.2.2–1.2.5). The survival time was expressed as the mean number of survival days postinoculation (dpi) for all the $PrP^{res}$-positive mice, with the standard error included. The attack rate was determined as the proportion of $PrP^{res}$-positive mice out of all the inoculated mice.

The Infective Dose50 ($ID_{50}$) of the Goat-BSE isolate (Foster et al., 1993; Goldmann et al., 1996) was assessed using the Reed-Muench method. The proportionate distance was calculated with the Reed-Muench formula ((percent of infected mice at dilution immediately above 50%) -50% / (percent of infected mice at dilution immediately above 50%) – (percent of infected mice at dilution immediately bellow 50%)) and applied to the dilution that produced the percentage of infection immediately above 50%. The infectious titer of the Goat-BSE isolate was expressed in $ID_{50}$ per gram ($ID_{50}$ $g^{-1}$) of tissue.

A regression curve was drawn with dilutions that produced 100% attack rates. The equation fit to the data was obtained ($y = e^{x}$) and used to determine the infectious titers in each different goat tissue from their survival time values.

### *2.3.2 Assesment of infectivity in goat tissues*

Brain, lymph node poplitealis, psoas major, and retractor bulbi muscles were obtained from WT ($R_{211}Q_{222}$/RQ), R/$Q_{211}$, and Q/$K_{222}$ goats orally inoculated with a Goat-BSE isolate (Foster et al., 1993; Goldmann et al., 1996) at the Institute for Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut (FLI), InselRiems, Germany. Samples were homogenized as 10% (w/v) in sterile 0.9 NaCl and some were pooled.

Groups of 6–9 individually identified BoPrP-Tg110 mice were intracerebrally inoculated with 20µl of each tissue homogenate and monitored for the presence of neurological signs. Euthanasia, tissue sampling, and brain $PrP^{res}$ detection by WB analysis were performed as previously described (see sections 1.2.2–1.2.5).

The relative infectivity of the different goat tissues was calculated as a function of the survival times obtained after their inoculation in BoPrP-Tg110 mice. Hence, survival times were interpolated into the equation fitting the data ($y = e^x$) previously obtained in the goat-BSE inoculum titration (see section 2.3.1).

## 3. Statistical analysis

A non-parametric Mann-Whitney-U test was applied to establish statistically significant differences in survival times of the $M_{142}$-Tg541 and $K_{176}$-Tg570 mouse lines and of the Wt-Tg501 mouse line inoculated with the same scrapie and BSE isolates. Statistical analyses were performed using PAST software (PAlaeontological STatistics, version 1.81). A difference of $P<0.05$ was considered as significant.

## 4. Buffers

- 4% 2-formaldehyde: 10% formaldehyde (w/v) (Carlo Erba 310358), 0.4% (w/v) $NaH_2PO_4$-$H_2O$ (Carlo Erba 480086), 0.4% (w/v) $Na_2HPO_4$ (Carlo Erba 480141), 0.4% (w/v) $NaHO_4$ N in $H_20$.
- BSA blocking buffer: 2% (w/v) Bovine Serum Albumin (BSA) fraction V (Sigma-Aldrich) in PBST.

- Eosin: 10% (w/v) Eosine Y (RAL 312717), 10% (w/v) Erythrosine 239 for histology (RAL 312827) in distilled $H_2O$.
- Extraction buffer: 1% (w/v) NP40, 1% (w/v) sodium deoxycholate, 10mM EDTA in PBS (pH 7.4) + complete™ cocktail of protease inhibitors (Roche).
- Laemmli buffer: 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 125mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 0.01% (w/v) bromofenol blue in H2O.
- Loading buffer: 50mM ditiotreitol (DTT), 1.5% Sodium Dodecyl Sulfate (SDS), 125mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 0.01% (w/v) bromofenol blue in $H_2O$.
- PBS + IP: 1X Phosphate Buffered Saline (PBS) + complete™ cocktail of protease inhibitors (Roche).
- Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 8.5 mM $Na_2HPO_4$ (pH 7.4) in $H_2O$.
- Phosphate Buffered Saline with Tween (PBST): PBS, 0.1% (v/v) Tween 20 (Merck 822184).
- TE: 10 mM Tris (pH 7.4), 0.1 mM EDTA.
- Transferring buffer: 25mM Tris, 192mM glycine, 10% (v/v) metanol in $H_2O$.
- Running buffer (for 12% polyacrylamide gels): 1.6% (v/v) acrylamide, 0.8 M Tris (pH 6.8), 5% (w/v) SDS, 5% (w/v) PSA and 0.2% (v/v) TEMED in $H_2O$.
- Saturation buffer: PBST, 0.2% BSA 30% (Sigma Aldrich A3299).
- Stacking buffer (for 12% polyacrylamide gels): 2% (v/v) acrylamide (BioRad), 13 mM Tris (pH 6.8), 1% (w/v) SDS, 1% (w/v) ammonium persulfate (PSA) and 0.1% (v/v) Tetramethylethylenediamine (TEMED) (BioRad) in $H_2O$.

# VI. RESULTS

# 1. Assessment of the resistance/susceptibility of polymorphic variants of the prion protein to prion infection in transgenic mice

## 1.1. Generation of transgenic mice expressing different goat and sheep $PrP^C$ polymorphic variants

### *1.1.1. Obtaining mouse founders*

Transgenic mouse lines (Tg) expressing either the goat wild type $PrP^C$ (Wt-Tg501 mice) or the $K_{222}$-$PrP^C$ variant ($K_{222}$-Tg516 mice) were previously generated by our group and the results related to their generation are included here for the purposes of comparison. In the present study, Tg lines expressing the $M_{142}$, $H_{154}$, $R_{171}$, $K_{176}$, or $Q_{211}$-$PrP^C$ polymorphic variants were new-generated following the same procedure used for obtaining goat wt- and $K_{222}$-$PrP^C$ mice.

The Wt-Tg501 mouse line was obtained from the DNA of a wild type goat whose *prnp* was identical to that of a goat previously reported (GenBank accession number AF117316.1). Its ORF was subcloned into the pGEM-T Easy Vector System (Promega) and later inserted into a MoPrP.Xho plasmid (Borchelt et al., 1996) containing the murine PrP promoter, and exon 1, intron 1, exon 2, and 3' untranslated sequences, and named as pMo-**Go**PrP.Xho (see Materials and Methods section, Fig. 9).

Transgenes containing different polymorphic variants of the *prnp* ($M_{142}$, $H_{154}$, $R_{171}$, $K_{176}$, $Q_{211}$, and $K_{222}$) were generated by directed mutation of the aforementioned pMo-**Go**PrP.Xho plasmid with specific primers (see Table 3 in Materials and Methods section). Mutations consisted of single nucleotide substitutions that gave rise to amino acid exchanges (Fig. 11). In this way, all these transgenes harbor the same $PrP^C$ sequence except for a single amino acid exchange corresponding to the desired $PrP^C$ polymorphic variant (see Materials and Methods section, Fig. 9).

Finally, these DNA constructions were purified and microinjected into pronuclear stage embryos collected from super-ovulated B6CBAF1 females in collaboration with Dr. Belén Pintado at the Centro Nacional de Biotecnología (CNB-CSIC). These female mice were mated with 129/Ola males carrying a null mutation in their endogenous PrP (Manson et al., 1994), and their progeny were

analyzed by PCR for the presence of the goat or sheep transgene (Table 8). Those carrying the transgene (founders) were selected for further characterization.

**Table 8.** Summary of the generation of the different transgenic mouse lines

| Transgene | Microinjected embryos | Mice born | Transgene positive mice (Founders) | Transgenesis rate (%) | Transgenic mouse lines |
|---|---|---|---|---|---|
| **WT[a]** | 667 | 41 | 6 | 14.6 | G500<br>G**501**<br>G502<br>G503<br>G504<br>G505 |
| **K222[a]** | 503 | 30 | 4 | 13.3 | G514<br>G515<br>**G516**<br>G517 |
| **M142** | 440 | 57 | 7 | 12.28 | G540<br>**G541**<br>G542<br>G543<br>G544<br>G545<br>G546 |
| **H154** | 560 | 39 | 6 | 15.38 | G560<br>G561<br>G562<br>**G563**<br>G564<br>G565 |
| **Q211** | 1049 | 85 | 3 | 3.53 | **G580**<br>G581<br>G582 |
| **R171** | 379 | 25 | 2 | 8 | G550<br>**G552** |
| **K176** | 262 | 43 | 4 | 9.30 | **G570**<br>G571<br>G572<br>G574 |

[a] Transgenic mouse lines expressing the WT $PrP^C$ or the $K_{222}$-$PrP^C$ variant were previously generated by our group and are included for comparison.

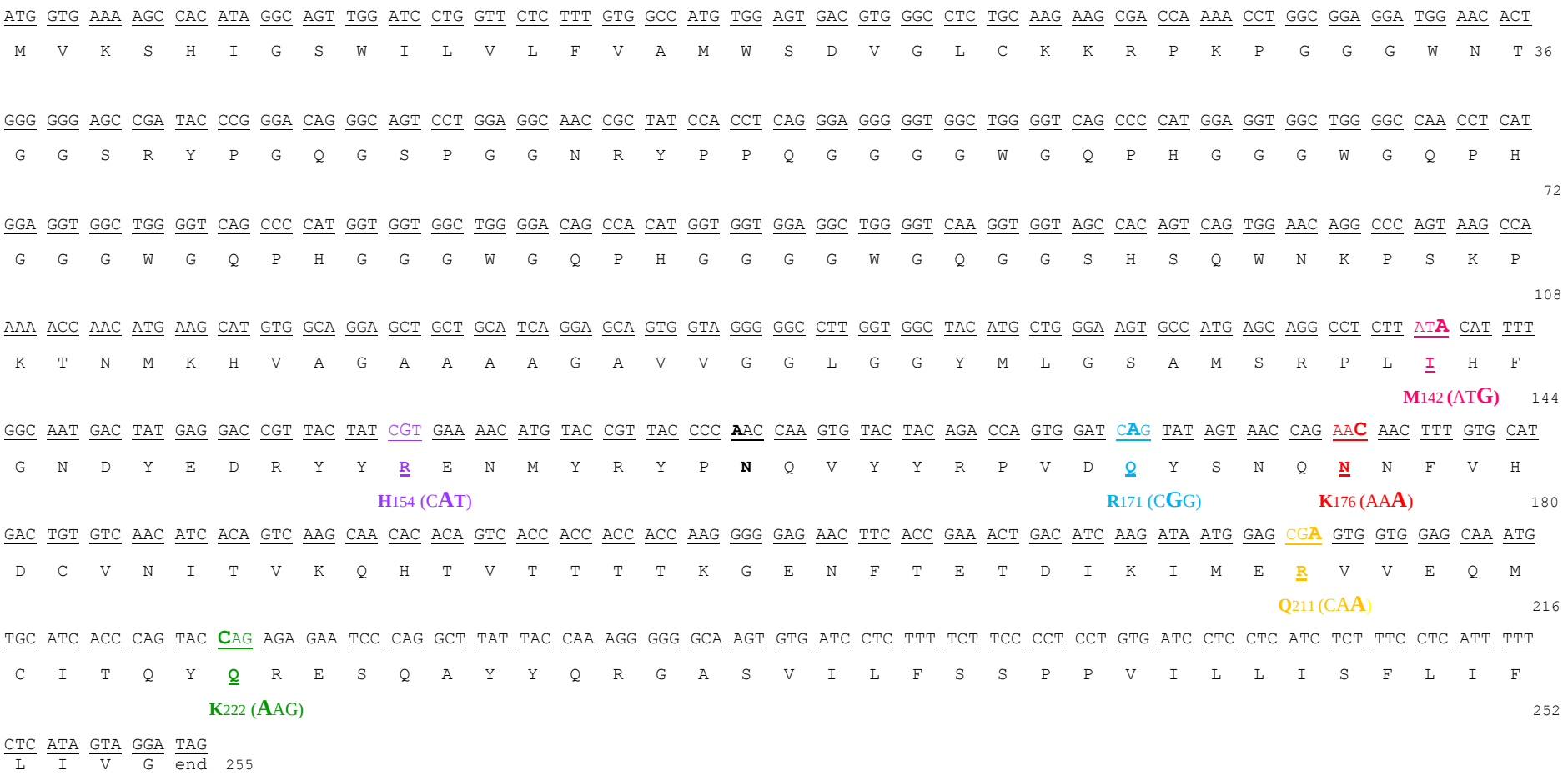


**Figure 11. Nucleotide sequence of the open reading frame (ORF) encoding the goat wild type prion protein (*Capra hircus* tissue-type PBL prion protein (PrP) gene; GENBANK ACCESSION NUMBER: AF117316.1); the polymorphic variants studied in this work are highlighted.**

### *1.1.2. Selection of mouse founders*

Several mouse lines (founders) were obtained for each transgene (Table 8). Founder animals also expressing the endogenous murine *prnp* gene (*prnp* mu+/- *go*+/-) were crossed with *prnp* null mice (*prnp* mu-/-) (Manson et al., 1994) to obtain transgene hemizygous lines in a murine *prnp* null background (*prnp* mu-/- *go*+/-), thereby preventing any possible interference by the endogenous murine $PrP^C$ in the susceptibility of goat and sheep Tg mice to TSEs (Kobayashi et al., 2009).
For each mouse line, transgene transmission ability, $PrP^C$ level of expression in the brain, and animal health and physiological behavior were analyzed (Table 9) in order to select the most suitable founders for the transmission studies.

All the offspring obtained from breeding the founders with *prnp* null mice (*prnp* mu-/-) (Manson et al., 1994) were analyzed by PCR to confirm the presence of the transgene. The majority of the founders were able to transmit the transgene to their progeny (Table 8), which, following Mendel´s principle of segregation (≤50% of transgene transmission when hemizygous founders were crossed with *prnp* null mice), thus suggests the existence of a single transgene insertion site in the mice genome. Founders with transgene transmission rates over 50% (indicative of multiple insertion sites), were discarded.
Then, the $PrP^C$ level of expression in the brain was determined in those mouse lines that transmitted the transgene (Table 9). Several animals from each progeny were sacrificed at 60 days of age and their whole brains were sampled. The amount of $PrP^C$ in their brains was assessed by Western blot (WB) and compared to the $PrP^C$ expressed in the brain of a wt goat or sheep (Fig. 12). Given that a directly proportional influence of the $PrP^C$ level of expression in the TSEs incubation times has been described (Castilla et al., 2003), mouse lines expressing the brain $PrP^C$ at goat and sheep physiological levels were selected in order to mimic as closely as possible the prion infection in goat and sheep.

**Table 9.** Summary of the characterization of the different transgenic mouse lines

| Trans gene | Tg mouse lines | Transgene transmission ability | Brain $PrP^C$ level of expression in hemizygosis[b] | Animal health and behavior |
|---|---|---|---|---|
| **WT[a]** | G500 | + | 1X | Normal |
| | **G501** | **+** | **1X** | **Normal** |
| | G502 | + | 0.75X | Normal |
| | G503 | + | 0.75X | Normal |
| | G504 | + | 0.75X | Normal |
| | G505 | + | 0.75X | Normal |
| **K222[a]** | G514 | **+** | 0.5X | Normal |
| | G515 | + | 1X | Normal |
| | **G516** | **+** | **1X** | **Normal** |
| | G517 | + | 0.25X | Normal |
| **M142** | G540 | + | 0.5X | Normal |
| | **G541** | **+** | **1X** | **Normal** |
| | G542 | + | 0.5X | Normal |
| | G543 | - | NA | Normal |
| | G544 | + | 0.25X | Normal |
| | G545 | + | 0.25X | Normal |
| | G546 | - | NA | Normal |
| **H154** | G560 | **+** | 0.25X | Normal |
| | G561 | **+** | 0.25X | Normal |
| | G562 | **-** | NA | Normal |
| | **G563** | **+** | **0.25X** | **Normal** |
| | G564 | **+** | 0X | Normal |
| | G565 | **+** | 0X | Normal |
| **Q211** | **G580** | **+** | **0.5X** | **Normal** |
| | G581 | - | NA | Normal |
| | G582 | - | NA | Normal |
| **R171** | G550 | + | 1X | Normal |
| | **G552** | **+** | **1X** | **Normal** |
| **K176** | **G570** | **+** | **2X** | **Normal** |
| | G571 | -[c] | NA | Normal |
| | G572 | + | 0.25X | Normal |
| | G574 | + | 0.75X | Normal |

[a] Characterization of the transgenic mouse lines expressing the WT $PrP^C$ or the $K_{222}$-$PrP^C$ variant that were previously generated by our group, and are here included for comparison.

[b] Brain $PrP^C$ expression in hemizygous Tg mouse lines related to that in goat or sheep brains.

[c] This founder had no progeny.

NA: Not analyzed.

A similar $PrP^C$ level of expression in the different Tg mouse lines was also pursued to ensure that the comparative study of their susceptibilities to prion infection was as accurate as possible. Thus, the $PrP^C$ expression in the brain of Wt-Tg501 and $K_{222}$-Tg516 (Aguilar-Calvo et al., 2014) was also compared with that of each of the new-generated transgenic mouse lines (Fig. 12A and D).

Brain $PrP^C$ electrophoretic profiles for all the goat and sheep Tg mouse lines were similar to those taken for goat and sheep brain $PrP^C$ (Fig. 12). $M_{142}$-Tg541 and $R_{171}$-Tg552 lines were selected on the basis of the similarity in the $PrP^C$ expression levels in their brains to those of goat, sheep, Wt-Tg501, and $K_{222}$-Tg516 mice brains (Fig. 12B and F). By contrast, we found no $K_{176}$ or $Q_{211}$ mouse line expressing their brain $PrP^C$ with similar levels to those of goat, sheep, Wt-Tg501, or $K_{222}$-Tg516 mice brains. Hence, $K_{176}$-Tg570 mouse line was selected due to its expression of the $PrP^C$, twice the $PrP^C$ in goat brains (2X) (Fig. 12G), which is comparable to the expression of Wt-Tg501 mice in homozygosis. $Q_{211}$-Tg580 mouse line was chosen because it was the only $Q_{211}$ founder able to transmit the transgene to their progeny, even if its expression was slightly lower than in Wt-Tg501 mice (Fig. 12D). Finally, $H_{154}$-Tg563 was used for transmission studies even though its $PrP^C$ expression in the brain was four times lower than in the goat brain (0.25X) (Fig. 12C).

All the founders and a number of their progeny were monitored until the end of their lifespans to see whether any health or behavior abnormalities developed. None of these animals showed any sign of disease or physiological alteration. Neither were any histological lesions suggestive of prion disease found in their brains using Hematoxylin-Eosin staining (HE) or Paraffin-embedded Tissue blots (PET-Blot). Therefore, spontaneous disease development due to the expression of the mutated $PrP^C$ could also be ruled out for all the transgenic mouse lines (Table 9).

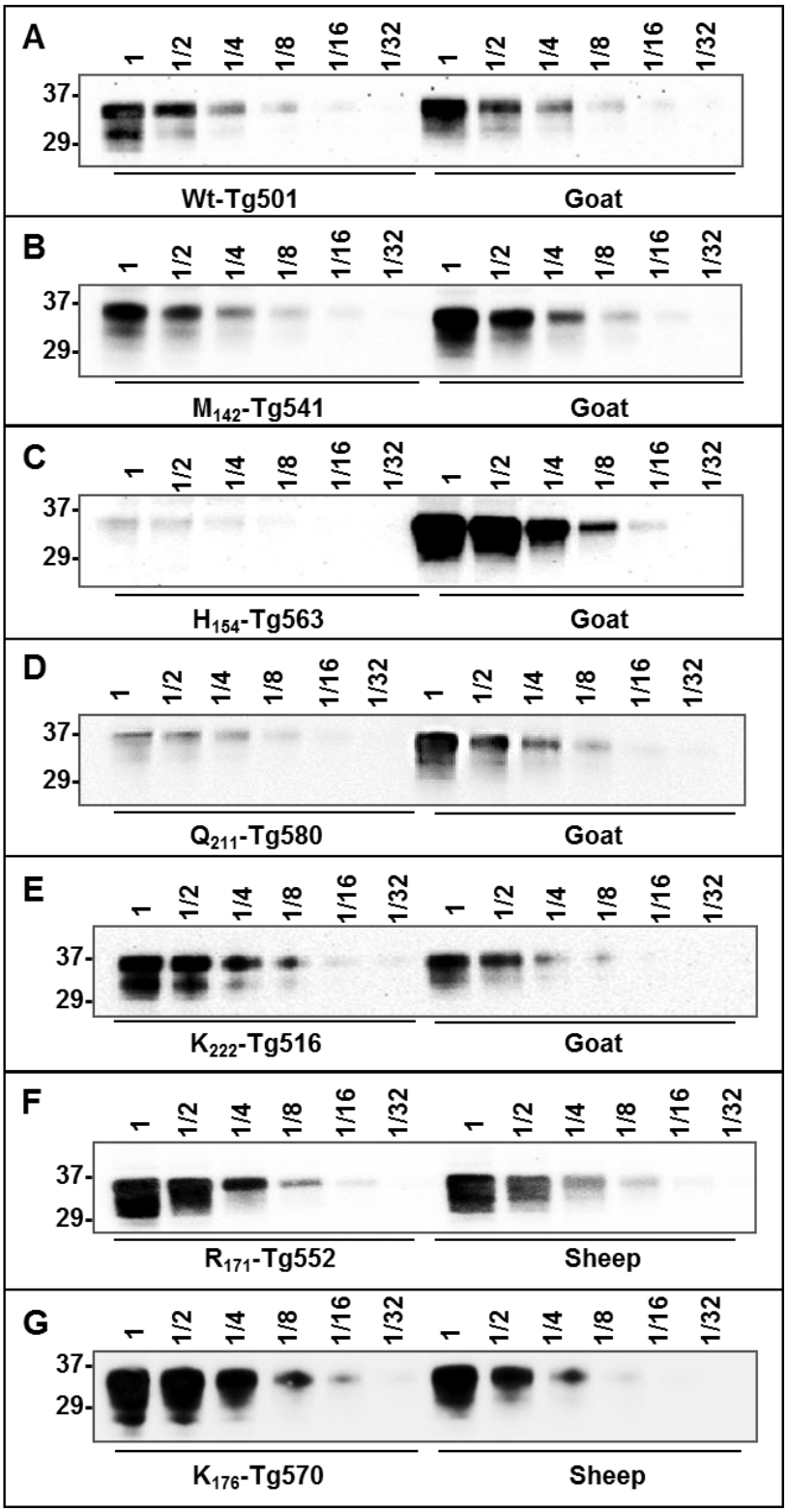


**Figure 12. Brain PrP$^C$ expression in hemizygous goat and sheep PrP transgenic mouse lines in comparison to goat and sheep brains.** Immunoblotting of brain PrP$^C$ of Wt-Tg501 (A), $M_{142}$-Tg541 (B), $H_{154}$-Tg563 (C), $Q_{211}$-Tg580 (D), $K_{222}$-Tg516 (E), $R_{171}$-Tg552 (F), and $K_{176}$-Tg570 (G) mice detected with 12B2 mAb. Direct sample (10% brain homogenates) and ½ serial dilutions were loaded on 12% Bis-Tris gels. The figure illustrates a representative set of three independent experiments. The relative molecular mass in kilodaltons is shown on the left-hand side of each blot.

In conclusion, $M_{142}$-Tg541, $H_{154}$-Tg563, $Q_{211}$-Tg580, $R_{171}$-Tg552, and $K_{176}$-Tg570 Tg mouse lines, all expressing similar levels of the same goat wt $PrP^C$ as Wt-Tg501 mice but with a single amino acid exchange, were generated in this work (Fig. 13). These mouse lines, together with previously generated Wt-Tg501 and $K_{222}$-Tg516 mice, were then used for transmission studies with a wide variety of scrapie and BSE isolates in order to study the role of each $PrP^C$ polymorphic variant on the resistance/susceptibility to prion infection.

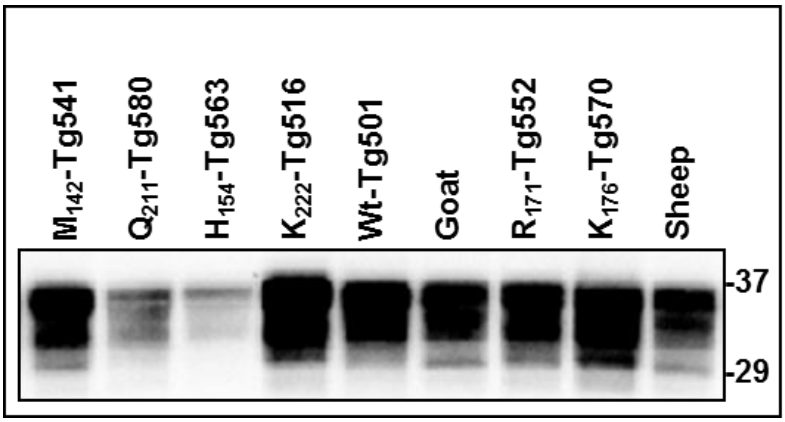


**Figure 13**. B**rain $PrP^C$ expression level of the different transgenic mouse lines in comparison to goat and sheep brains.** Immunoblotting of brain $PrP^C$ from $M_{142}$-Tg541, $Q_{211}$-Tg580, $H_{154}$-Tg563, $K_{222}$-Tg516, Wt-Tg501, $R_{171}$-Tg552 and $K_{176}$-Tg570 mice, goat, and sheep detected with 12B2 mAb. Identical amounts of brain material (10% brain homogenate) were loaded on 12% Bis-Tris gels. The relative molecular mass in kilodaltons is given on at the right-hand side of the blot.

## 1.2 Susceptibility of goat wild type-PrP$^{C}$ transgenic mice to a wide diversity of prion isolates

Goat Wt-Tg501 mice were previously generated and initially challenged with a limited number of scrapie isolates by our group. These mice resulted fully susceptible to these scrapie transmissions, thereby suggesting that this mouse line is a promising tool for studying this infectious agent in goats and sheep. However, the low number of isolates tested prevented us from drawing further conclusions at this point.In order to fully assess the susceptibility of this mouse line to the prion infection, Wt-Tg501 mice were challenged with a broad panel of scrapie and BSE isolates (see Tables 4-7 in Materials and Methods for more information).

### 1.2.1 Characteristics of prion isolates

A collection of scrapie isolates were selected on the basis of heterogeneity according to the following factors: i) geographical origin, ii) PrP$^{Sc}$ amino acid sequence, iii) brain PrP$^{res}$ glycoprofile, and iv) transmission features in BoPrP-Tg110 mice. Table 10 summarizes the characteristics of the scrapie isolates. Scrapie isolates were obtained from numerous outbreaks from different European countries (see Tables 4-6 in Materials and Methods for more information). All the isolates were sequenced to determine the PrP$^{Sc}$ amino acid sequence according to the procedures described in Materials and Methods, section 1.2.1.1. The polymorphic variants found in the isolates are given in Table 10, column "PrP$^{Sc}$ genotype".

Brain PrP$^{res}$ glycoprofile for all the scrapie isolates were analyzed by Western blot (WB) following the procedures described in Materials and Methods, section 1.2.1.2. The apparent molecular mass of the PrP$^{res}$ unglycosilated band of each isolate is given in kilodaltons (K) in Table 10, column "PrP$^{res}$ profile". Most of the classical scrapie isolates exhibited a single 21K PrP$^{res}$ unglycosilated band (Fig. 14A). Only Goat-Sc F14 and Goat-Sc UKB2 isolates were distinguishable by their double 19-21K PrP$^{res}$ unglycosilated band (Fig. 14A), thereby suggesting the presence of more than one scrapie agent in these isolates. In contrast, all atypical scrapie isolates showed a similar Nor98 PrP$^{res}$ WB glycoprofile characterized by a ladder pattern and a low unglycosilated band of around 7K (Fig. 14B).

**Table 10.** Characteristics of the panel of scrapie and BSE isolates

| | Isolate | | | | Transmission in BoPrP-Tg110 | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 1st passage | | 2nd passage | |
| | Name | Origin | $PrP^{Sc}$ genotype[a] | $PrP^{res}$ profile[b] | ST ± SEM ($n/n_0$)[c] | $PrP^{res}$ profile | ST ± SEM ($n/n_0$)[d] | $PrP^{res}$ profile |
| Classical scrapie | **Goat-Sc I2** | Italy | wt; S/S240 | 21K | 453 (1/6) | 21K | **167±9 (7/7)** | 21K |
| | **Goat-Sc I3** | | wt; P/P240 | 21K | 464 (1/7) | 21K | **163±16 (7/7)** | 21K |
| | **Goat-Sc I4** | | wt; R/Q211; S/P240 | 21K | >650 (0/5) | - | **NA** | NA |
| | **Goat-Sc I9** | | wt; R/H143; S/P240 | 21K | 324±90 (6/6) | 21K | **174±8 (5/5)** | 21K |
| | **Goat-Sc I12** | | wt; S/P240 | 21K | >650 (0/7) | - | **NA** | - |
| | **Goat-Sc F2** | France | wt; S/P240 | 21K | 343±163 (4/5) | 19K | **198±13 (6/6)** | 19K |
| | **Goat-Sc F3** | | wt; P/P240 | 21K | 290±48 (3/6) | 19K | **191±3 (6/6)** | 19K |
| | **Goat-Sc F6** | | wt; S/P240 | 21K | 523±166 (6/6) | 19K | **178±13 (5/5)** | 19K |
| | **Goat-Sc F10** | | wt; S/P240 | 21K | 556±68 (4/4) | 19K | **575±43 (5/5)** | 19K |
| | **Goat-ScF11** | | wt; I/M142; P/P240 | 21K | >650 (0/6) | - | **NA** | NA |
| | **Goat-ScF14** | | wt; I/M142; S/P240 | 19-21K | >650 (0/6) | - | **NA** | NA |
| | **Goat-Sc F16** | | wt; I/M142; S/P240 | 21K | 348±58 (3/6) | 21K | **177±9 (6/6)** | 21K |
| | **Goat-ScG2** | Greece | wt; P/P240 | 21K | 610 (1/6) | 19K | **348±85 (6/6)** | 19K |
| | **Goat-ScG3** | | wt; R/H143; P/P240 | 21K | >650 (0/6) | - | **NA** | - |
| | **Goat-Sc N1** | Netherlands | wt; R/H143; S/P240 | 21K | 649±9 (3/3) | 19K | **>450 (ongoing)** | - |
| | **Goat-Sc N2** | | wt; R/H143; S/P240 | 21K | >650 (0/6) | - | **NA** | - |
| | **Goat-Sc N3** | | wt; P/P240 | 21K | 324, 703 (2/6)* | 19K | **203±7 (5/5)** | 19K |
| | | | | | | | **493±72 (6/6)** | 19K |
| | **Goat-Sc S2** | Spain | wt; S/P240 | 21K | 384±149 (6/6) | 19K | **237±39 (9/9)** | 19K |
| | **Goat-Sc S3** | | wt; P/P240 | 21K | 271±19 (6/6) | 19K | **254±52 (4/4)** | 19K |
| | **Goat-Sc UKA2** | United Kingdom | wt; S/P240 | 21K | 255±69 (5/5) | 19K | **187±6 (6/6)** | 19K |
| | **Goat-Sc UKB2** | | wt; G/S127; P/P240 | 19-21K | 205±12 (7/7) | 19K | **196±5 (6/6)** | 19K |
| | **Goat-Sc UKD2** | | wt; G/S127; P/P240 | 21K | >650 (0/7) | - | **NA** | NA |
| | **Goat-Sc Zyp13** | Cyprus | wt; P/P240 | 21K | >650 (2/2) | 19K | **371±51 (6/6)** | 19K |
| | **Goat-Sc Zyp21** | | wt; P/P240 | 21K | >650 (2/5) | 19K | **387±12 (3/3)** | 19K |
| | **Sheep-Sc 21** | France | wt; S/S240 | 21K | 244±13 (6/6) | 19K | **187±2 (4/4)** | 19K |
| | **Sheep-Sc Langlade** | | wt; S/S240 | 21K | 504±47 (6/6) | 19K | **229±37 (4/4)** | 19K |
| | **Sheep-Sc 09** | | wt; F/F141;S/S240 | 21K | 230±66 (4/6) | 19K | **168±6 (6/6)** | 19K |
| Atypical scrapie | **Goat-Sc I15** | Italy | wt; H/R154;S/P240 | Nor98[e] | **>650 (1/6)** | 19K | NA | NA |
| | **Sheep-Sc engavagen** | Norway | wt; F/F141;S/S240 | Nor98 | **395±44 (3/5)** | 19K | NA | NA |
| | **Sheep-Sc M45** | Spain | wt; S/S240 | Nor98 | **>650 (0/6)** | - | NA | NA |
| | **Sheep-Sc TOA4** | | wt; H/Q171; S/S240 | Nor98 | **>650 (0/6)** | - | NA | NA |
| BSE | **Goat-BSE1** | France | wt S/S240 | 19K | 255±7 (5/5) | 19K | **242±3 (5/5)** | 19K |
| | **Goat-BSE2** | | wt P/S240 | 19K | 289±24 (6/6) | 19K | **277±10 (6/6)** | 19K |
| | **Sheep-BSE** | | wt; S/S240 | 19K | 246±9 (5/5) | 19K | **237±5 (5/5)** | 19K |
| | **Cattle-BSE** | | Cattle wt | 19K | 282±41 (6/6) | 19K | **245±26 (5/5)** | 19K |
| | **Cattle-BSE L** | | Cattle wt | 19K-L[f] | 214±15 (6/6) | 19K-L | **188±1 (6/6)** | 19K-L |

See next page for footnotes

[a] Scrapie isolates were sequenced following the procedures described in Materials and Methods, section 1.2.1.1. Wild type (wt) $PrP^{Sc}$ sequence ($A_{136}R_{154}Q_{171}$/ $A_{136}R_{154}Q_{171}$) and the polymorphic variants observed are indicated.

[b] Brain $PrP^{res}$ was detected by Western blot using Sha31 or 9A2 mAb (Fig. 14–15). The apparent molecular mass of the $PrP^{res}$ unglycosilated band is given in kilodaltons (K).

[c] n/n0: diseased, $PrP^{res}$: positive/inoculated animals.

[d] Isolates were considered fast (in red) if survival times were <300 days post-inoculation (dpi) at second passage, or slow (in blue) when survival times were >300 dpi at second passage.

[e] Nor98: $PrP^{res}$ Western blot glycoprofile characterized by a ladder pattern and a low unglycosilated band of around 7K.

[f] 19K-Ltype: $PrP^{res}$ Western blot glycoprofile characterized by a predominant monoglycosylated moiety and a smaller molecular size of the nonglycosylated fragment compared to the classical BSE agent.

* Among the Goat-Sc N3 infected BoPrP-Tg110 mice one exhibited very long survival times of 703 dpi. The brain from this animal was further passaged in BoPrP-Tg110 mice (in blue) separately from the other mouse (in red).

Orange cells: isolates transmitted with no apparent transmission barrier.

Green cells: isolates transmitted with a moderate-to-high transmission barrier.

White cells: isolates not transmitted.

NA: Not analyzed

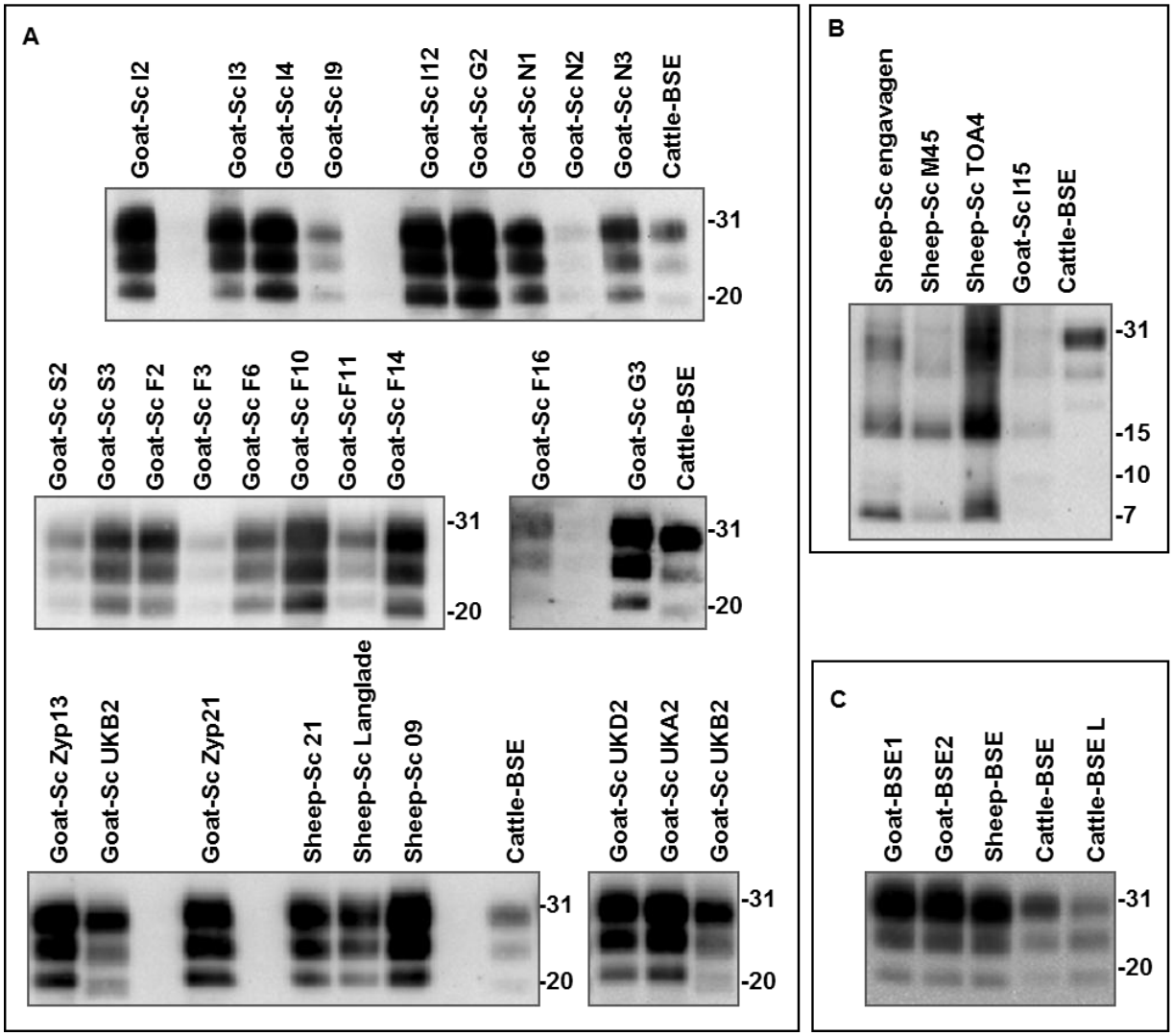


**Figure 14. $PrP^{res}$ of scrapie and BSE isolates.** Immunoblots of brain $PrP^{res}$ detected in classical scrapie (A), atypical scrapie (B), and BSE (C) isolates. $PrP^{res}$ was detected with Sha31 mAb for classical scrapie and BSE isolates, and with 9A2 mAb for atypical scrapie isolates. Similar quantities of sample (10% brain homogenate) were loaded for adequate comparison. Molecular weights in kilodaltons are shown on the right-hand side of the blot.

Table 10 also shows the results of the prion transmission experiments in BoPrP-Tg110 mice, and includes survival times (ST) and attack rates (AR) at first and second passage, and brain $PrP^{res}$ WB profile. These transmission studies were conducted in the framework of the StrepBSE in goat EU project (UE-FP6-2005-FOOD4B-036353) and permitted us to analyze the behavior of scrapie isolates in the bovine species context.

For these transmission experiments, groups of 6–9 BoPrP-Tg110 mice were intracerebrally (IC) inoculated with the complete panel of prion isolates described above. When the progression of the disease was evident or at the end of their lifespan (≈650 days), BoPrP-Tg110 mice were euthanized and their brains sampled for detecting the presence of $PrP^{res}$ using Sha31 mAb (Fig. 15), following the procedures described in Materials and Methods, section 1.2.1.2.

Brain homogenates from mice scoring positive for $PrP^{res}$ were used for further passaging. When all mice scored negative for $PrP^{res}$ on primary passage, $PrP^{res}$-negative brain homogenates were used for second passage (see Materials and Methods, section 1.2.1). ST and AR were assessed following the procedures described in Materials and Methods, section 1.2.4 to determine the susceptibility of BoPrP-Tg110 mice to scrapie infection. ST are expressed as the mean number of survival days postinoculation (dpi) for all the $PrP^{res}$-positive mice, including the standard error (SM), while AR were assessed as the proportion of $PrP^{res}$-positive mice out of all the inoculated mice.

Taking into account the transmission efficiency in BoPrP-Tg110 mice at first and second passage, classical scrapie isolates can be classified in three groups:

i) Classical scrapie isolates transmitted to BoPrP-Tg110 mice with low or no apparent transmission barrier. Seven out of the 27 isolates showed 100% AR at first passage and a weak decrease in their ST after second passage in these bovinized Tg mice (in orange in Table 9), suggesting a low or no transmission barrier in BoPrP-Tg110 mice.

ii) Classical scrapie isolates transmitted to BoPrP-Tg110 mice with a moderate-to-high apparent transmission barrier**.** Thirteen out of the 27 isolates showed less than 100% AR and/or very long ST at first passage, but were fully transmitted (100% AR)

to these bovinized Tg mice at second passage (in green in Table 10). This drastic decrease in ST is suggestive of a high transmission barrier.

iii) Classical scrapie isolates that were not transmitted to BoPrP-Tg110 mice. BoPrP-Tg110 mice did not succumb to the primary transmission of seven out of the 27 classical scrapie isolates tested (in white in Table 10), suggesting that an absolute transmission barrier for these agents exists in this mouse model. However, low infectious titers cannot be ruled out as the cause of these negative results in these cases, in which no second passage was performed.

All the atypical scrapie isolates had poor transmission efficiencies at primary passage in BoPrP-Tg110 mice showing long ST and/or low AR (Table 9). Moreover, two out of the four isolates inoculated were not transmitted at first passage.

On the basis of the $PrP^{res}$ glycloprofile, two kinds of classical scrapie isolates were observed: those that showed a 21K $PrP^{res}$ unglycosilated band upon passage in BoPrP-Tg110 mice, and those that produced a 19K $PrP^{res}$ unglycosilated band in this mouse model (Fig. 15A). Although all Italian scrapie isolates exhibited a 21K $PrP^{res}$ unglycosilated band in BoPrP-Tg110 mice, only one non-Italian isolate (Goat-Sc F16) shared this property, a finding that thus supports the idea of a regional distribution of scrapie strains. The rest of the panel produced a 19K $PrP^{res}$ unglycosilated band regardless of their geographical origin. Contrary to classical scrapie isolates, atypical scrapie isolate switched its Nor98 $PrP^{res}$ glycoprofile to a classical three banded $PrP^{res}$ pattern undistinguishable from classical BSE upon passage in BoPrP-Tg110 mice (Fig. 15B).

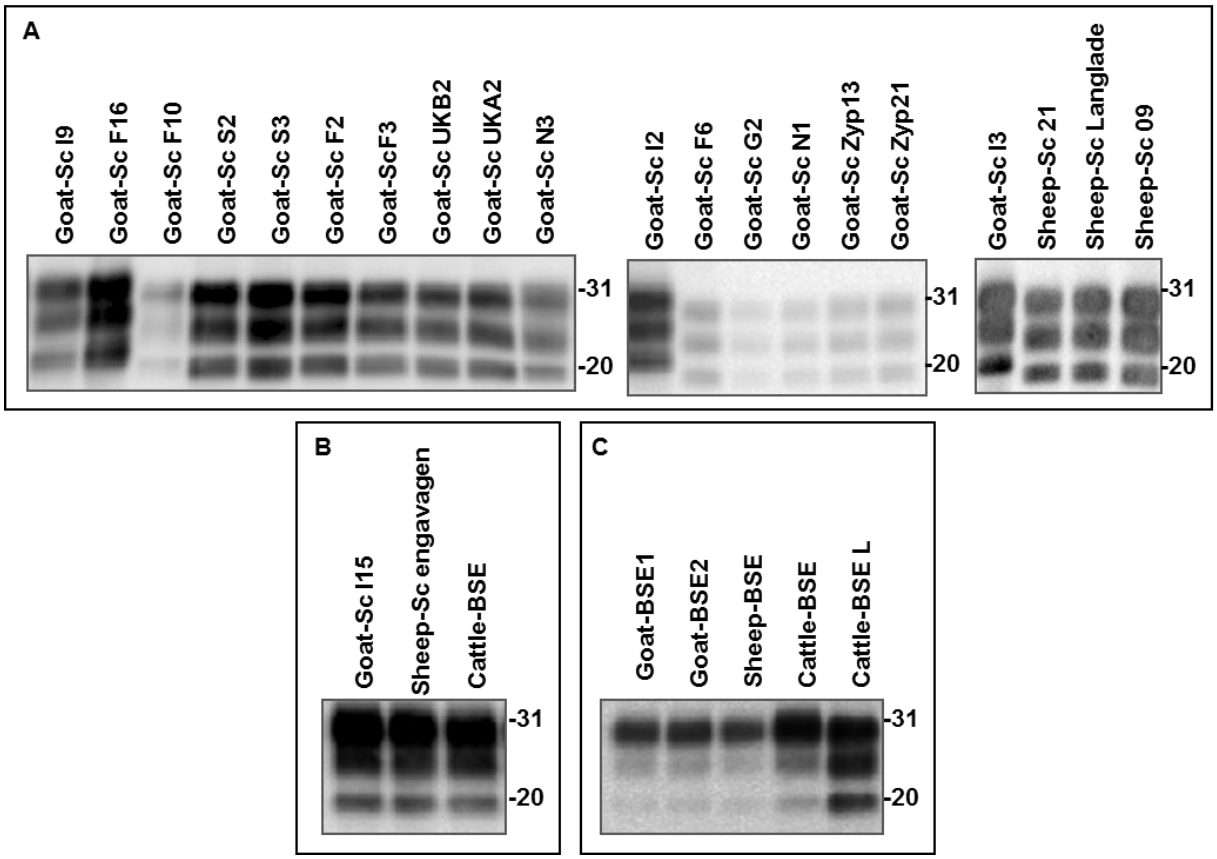


**Figure 15. PrP$^{res}$ of scrapie and BSE isolates after transmission in BoPrP-Tg110 mice.** Immunoblots of brain PrP$^{res}$ from BoPrP-Tg110 mice infected with classical scrapie (A), atypical scrapie (B), and BSE, BSE L isolates (C) detected with Sha31 mAb. Similar quantities of sample (10% brain homogenate) were loaded for adequate comparison. Molecular weights in kilodaltons are shown on the right-hand side of the blot.

Finally, given the ST at second passage in BoPrP-Tg110 mice, this panel of scrapie isolates could be classified as either fast (<300 dpi, in red in Table 10) or slow (>300 dpi, in blue in Table 10). Intriguingly, all the isolates that produced a 21K PrP$^{res}$ unglycosilated band in BoPrP-Tg110 mice (Italian isolates and Goat-Sc F16) were also similar in terms of the other transmission features in BoPrP-Tg110 mice, i.e. they all exhibited a moderate transmission barrier and behaved as fast strains**.** For the rest of the panel, fast and slow isolates could be identified independently of their geographical origin.

Besides this panel of scrapie isolates, three BSE isolates naturally or experimentally obtained from cattle, sheep, or goat species (see Table 7 in Materials and Methods for more information) were selected to study the susceptibility of Wt-Tg501 mice to BSE infection. In addition, a natural case of an atypical BSE agent, cattle BSE L, was also tested to analyze its transmission features in the goat context.

As previously reported (Castilla et al., 2005a; Castilla et al., 2003; Espinosa et al., 2007a), all the BSE isolates were transmitted to BoPrP-Tg110 mice that had no transmission barrier (Table 11). Brain $PrP^{res}$ WB glycoprofiles of both classical and atypical BSE isolates were maintained in these bovinized Tg mice (Fig. 14C and 15C). Cattle, sheep, and goat-BSE isolates produced a characteristic BSE-like WB profile characterized by a predominant diglycosilated band and an apparent molecular mass of the unglycosilated band of 19K. By contrast, the atypical BSE L isolate showed a $PrP^{res}$ "light pattern", with a lower molecular weight than classical BSE and no predominant band (Figs. 14C and 15C).

### *1.2.2 Prion transmission features in transgenic mice expressing goat wild type-$PrP^{C}$*

Groups of 6–9 goat Wt-Tg501 mice were IC inoculated with the complete panel of prion isolates described above. When the progression of the disease was evident or at the end of their lifespan (≈650 days), the Wt-Tg501 mice were euthanized and their brains sampled for both detecting the presence of $PrP^{res}$ by WB analysis and studying the $PrP^{Sc}$ deposition and histopathological alterations using Hematoxylin-Eosin staining (HE) and Paraffin-embedded Tissue blots (PET-Blot), following the procedures described in Materials and Methods, section 1.2.6). Brain homogenates from $PrP^{res}$ positive mice were used for further passaging. ST and AR were assessed as previously described (see section 1.2.4 in Materials and Methods) to determine the susceptibility of the Wt-Tg501 mouse line to the prion infection. As well, 6–7 Wt-Tg501 mice were IC inoculated with healthy goat brains to act as controls for the appearance of a spontaneous prion disease.

### *1.2.2.1 Survival times and attack rates in Wt-Tg501 mice*

Homozygous Wt-Tg501 mice were fully susceptible (100% AR) to the primary transmission of all the classical scrapie isolates except for two isolates (Goat-Sc G2 and Goat-Sc UKB2) which displayed 13% and 20% AR, respectively. ST greatly varied at first passage, ranging from 200 to 650 dpi (Table 11). These differences in transmission efficiencies could be due to: i) differences in infectious titers, ii) PrP amino acid differences between host and donor, or iii) transmission strain differences. To study further the origin of these differences, second passages were performed (Table 11). After second passage, Wt-Tg501 mice were fully susceptible (100% AR) to the whole panel of classical scrapie isolates (Table 11). A reduction in ST was observed in some isolates (in green in Table 11), thereby suggesting the presence of an apparent transmission barrier. In terms of the ST at second passage, fast (<300 dpi, in red in Table 12) and slow (>300 dpi, in blue in Table 11) scrapie isolates could be distinguished. Notably, Italian isolates differed from the rest of the isolates due to their very long ST (550-650dpi).

Atypical scrapie isolates were also fully transmitted (100% AR) to Wt-Tg501 mice (Table 11). Despite the high level of expression of homozygous Wt-Tg501 mice (twice the $PrP^{C}$ in the goat brain), all inoculated mice exhibited very long ST >400 dpi.

Homozygous Wt-Tg501 mice were also fully susceptible (100% AR) to the inoculation with all the BSE isolates tested. Mice had very similar ST of around 350-400 dpi regardless of the BSE species of origin—cattle, sheep, or goat (Table 11). Moreover, cattle-BSE registered very similar ST at second passage, thereby suggesting a low transmission barrier to the classical cattle-BSE in Tg mice expressing goat wt-$PrP^{C}$. Atypical cattle BSE L agent was also transmitted to Wt-Tg501 mice with high efficiency (100% AR). However, ST were longer than those obtained with Cattle-BSE infections at first passage (Table 11), and clearly decreased after second passage which indicates the presence of an apparent transmission barrier to Cattle-BSE L.

**Table 11.** Transmission of scrapie and BSE isolates to goat Wt-Tg501 mice

| | Isolate | | | | Transmission in Wt-Tg501 mice | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 1st passage | | 2nd passage | |
| | Name | Origin | $PrP^{Sc}$ genotype[a] | $PrP^{res}$ profile[b] | ST ± SEM ($n/n_0$)[c] | $PrP^{res}$ profile | ST ± SEM ($n/n_0$)[d] | $PrP^{res}$ profile |
| Classical scrapie | **Goat-Sc I2** | Italy | wt; S/S240 | 21K | 526±123(6/6) | 21K | **>600 (ongoing)** | - |
| | **Goat-Sc I3** | | wt; P/P240 | 21K | >650 (5/5) | 21K | **>600 (ongoing)** | - |
| | **Goat-Sc I4** | | wt; R/Q211; S/P240 | 21K | 582±20 (6/6) | 21K | **>350 (ongoing)** | - |
| | **Goat-Sc I9** | | wt; R/H143; S/P240 | 21K | 578±25 (5/5) | 21K | **547±25 (6/6)** | 21K |
| | **Goat-Sc I12** | | wt; S/P240 | 21K | 591±42 (4/4) | 21K | **>350 (ongoing)** | - |
| | **Goat-Sc F2** | France | wt; S/P240 | 21K | 259±28 (4/4) | 21K | **212±16 (6/6)** | 21K |
| | **Goat-Sc F3** | | wt; P/P240 | 21K | 287±14 (6/6) | 21K | **228±7 (6/6)** | 21K |
| | **Goat-Sc F6** | | wt; S/P240 | 21K | 468±15 (4/4) | 21K | **299±6 (6/6)** | 21K |
| | **Goat-Sc F10** | | wt; S/P240 | 21K | 449±19 (5/5) | 21K | **372±14 (6/6)** | 21K |
| | **Goat-Sc F11** | | wt; I/M142; P/P240 | 21K | >650 (6/6) | 21K | **>330 (ongoing)** | - |
| | **Goat-Sc F14** | | wt; I/M142; S/P240 | 19-21K | 526±46 (4/4) | 19-21K | **287±94 (4/4)** | 19-21K |
| | **Goat-Sc F16** | | wt; I/M142; S/P240 | 21K | 551±89 (3/3) | 21K | **242±26 (5/5)** | 21K |
| | **Goat-ScG2** | Greece | wt; P/P240 | 21K | >650 (1/4) | 21K | **>400 (ongoing)** | - |
| | **Goat-ScG3** | | wt; R/H143; P/P240 | 21K | 466±35 (4/4) | 21K | **375±25 (4/4)** | 21K |
| | **Goat-Sc N1** | Netherlands | wt; R/H143; S/P240 | 21K | 535±8 (4/4) | 21K | **339±19 (5/5)** | 21K |
| | **Goat-Sc N2** | | wt; R/H143; S/P240 | 21K | 555±4 (4/4) | 21K | **>350 (ongoing)** | - |
| | **Goat-Sc N3** | | wt; P/P240 | 21K | 451±9 (4/4) | 21K | **254±50 (6/6)** | 21K |
| | **Goat-Sc S2** | Spain | wt; S/P240 | 21K | 228±15 (6/6) | 21K | **233±4 (6/6)** | 21K |
| | **Goat-Sc S3** | | wt; P/P240 | 21K | 221±16 (6/6) | 21K | **233±64 (5/5)** | 21K |
| | **Goat-Sc UKA2** | United Kingdom | wt; S/P240 | 21K | 245±36 (5/5) | 19-21K | **252±8 (6/6)** | 19-21K |
| | **Goat-Sc UKB2** | | wt; G/S127; P/P240 | 19-21K | 381 (1/5) | 19-21K | **214±34 (7/7)** | 19-21K |
| | **Goat-Sc UKD2** | | wt; G/S127; P/P240 | 21K | 547±100(5/5) | 21K | **>450 (ongoing)** | - |
| | **Goat-Sc Zyp13** | Cyprus | wt; P/P240 | 21K | 483±15 (4/4) | 21K | **301±10 (4/4)** | 21K |
| | **Goat-Sc Zyp21** | | wt; P/P240 | 21K | 475±31 (5/5) | 21K | **324±9 (4/4)** | 21K |
| | **Sheep-Sc 21** | France | wt; S/S240 | 21K | 194±5 (6/6) | 21K | **205±18 (6/6)** | 21K |
| | **Sheep-Sc Langlade** | France | wt; S/S240 | 21K | >650 (3/3) | 21K | **222±5 (6/6)** | 21K |
| | **Sheep-Sc 09** | France | wt; F/F141;S/S240 | 21K | 277±31 (5/5) | 21K | **NA** | NA |
| Atypical scrapie | **Goat-Sc I15** | Italy | wt; H/R154 S/P240 | Nor98[e] | **557±73(6/6)** | Nor 98 | NA | NA |
| | **Sheep-Sc NOR98/Tg338** | Norway | wt; V/V136; S/S240 | Nor98 | **601±50 (4/4)** | Nor 98 | NA | NA |
| | **Sheep-Sc engavagen** | France | wt; F/F141;S/S240 | Nor98 | **623±48 (6/6)** | Nor 98 | NA | NA |
| | **Sheep-Sc M45** | Spain | wt; S/S240 | Nor98 | **453±12 (5/5)** | Nor 98 | NA | NA |
| | **Sheep-ScTOA4** | | wt; Q/H 171; S/S240 | Nor98 | **407±17 (5/5)** | Nor 98 | NA | NA |
| BSE | **Goat-BSE1** | France | wt; S/S240 | 19K-BSE[f] | **366±24 (5/5)** | 19K-BSE | - | - |
| | **Goat-BSE2** | | wt; S/P240 | 19K-BSE | **346±16 (7/7)** | 19K-BSE | - | - |
| | **Sheep-BSE** | | wt; S/S240 | 19K-BSE | **405±37 (7/7)** | 19K-BSE | - | - |
| | **Cattle-BSE** | | Cattle wt | 19K-BSE | **357±9 (6/6)** | 19K-BSE | 326±26 (5/5) | 19K-BSE |
| | **Cattle-BSE L** | | Cattle wt | 19K-Ltype[g] | **581±36 (5/5)** | 19K-BSE | 300±41 (5/5) | 19K-BSE |
| | **Healthy goat brain** | France | wt; P/P240 | - | **>650 (0/6)** | - | NA | NA |

[a] Scrapie isolates were sequenced following the procedures described in Materials and Methods (see section 1.2.1.1). Wild type (wt) $PrP^{Sc}$ sequence ($A_{136}R_{154}Q_{171}/A_{136}R_{154}Q_{171}$) and the polymorphic variants observed are given.

[b] Brain $PrP^{res}$ was detected by Western blot using Sha31 or 9A2 mAb (Fig. 14-16). The apparent molecular mass of the $PrP^{res}$ unglycosilated band is given in kilodaltons (K).

[c] n/n0: diseased, $PrP^{res}$: positive/inoculated animals.

[d] Isolates were considered fast (in red) when survival times were <300 days post-inoculation (dpi) at second passage, or slow (in blue) when survival times were >300 dpi at second passage.

[e] Nor98: $PrP^{res}$ Western blot glycoprofile characterized by a ladder pattern and a low unglycosilated band of around 7K.

[f] 19K-BSE: $PrP^{res}$ Western blot glycoprofile characterized by a predominant diglycosylated moiety and a 19K unglycosylated band.

[g] 19K-Ltype: $PrP^{res}$ Western blot glycoprofile characterized by a predominant monoglycosylated moiety and a lower molecular size of the nonglycosylated fragment than in the classical BSE agent.

Orange cells: isolates transmitted with no apparent transmission barrier.
Orange cells: isolates transmitted with no apparent transmission barrier.
Green cells: isolates transmitted with a moderate-to-high transmission barrier.
NA: Not analyzed.

Finally, no evidence of prion disease was determined in Wt-Tg501 mice IC inoculated with healthy goat brain (Table 11). These mice were euthanized at 650 dpi without showing any clinical signs and their brains were scored as $PrP^{res}$ negative, which thus rules out any spontaneous development of a prion disease in this mouse line.

### *1.2.2.2 Brain $PrP^{res}$ Western blot profile*

Brains from all of the challenged Wt-Tg501 mice were routinely analyzed for the presence of $PrP^{res}$ using WB. Sha31 mAb was used for detecting brain $PrP^{res}$ in classical scrapie and BSE-challenged mice, whereas 9A2 mAb was used for detecting brain $PrP^{res}$ in atypical scrapie-infected animals. Their $PrP^{res}$ signature was then compared to those of the original isolates (Fig. 16).

In general, the electrophoretic motilities observed for the original prion isolates were conserved upon transmission to Wt-Tg501 mice regardless of whether they were 21K or 19K (Fig. 16) scrapie isolates. In particular, Goat-Sc UKB2-infected mice had in their brains the double 19-21K $PrP^{res}$ unglycosilated band observed in the corresponding original isolates (Fig. 16A). Intriguingly, inoculation with the Goat-Sc UKA2 isolate, which originally harbored a 21K $PrP^{res}$ unglycosilated bands, gave rise to variable $PrP^{res}$ WB glycoprofiles: some mice exhibited a 19-21K double unglycosilated band (Fig. 16A, Goat-Sc UKA2 in Tg501(1)), while in others a 21K single unglycosilated band was observed (Fig. 16A, Goat-Sc UKA2 in Tg501(2)). Goat-Sc F14-infected mice exhibited the double 19- 21K $PrP^{res}$ unglycosilated bands observed in the original isolate. These particular cases suggest the coexistence of two different agents in each of these isolates. $PrP^{res}$ WB profile of atypical scrapie isolates was also maintained upon transmission in goat Wt-Tg501 mice. In all cases, atypical $PrP^{res}$ was characterized by a ladder pattern with a low predominant band of around 7K (Fig. 16B).

On the other hand, WB analysis of brain-$PrP^{res}$ from goat-BSE isolates revealed a typical $PrP^{res}$ banding pattern, characterized by small fragments (19K fragment for the unglycosylated-band) and prominent diglycosylated species. This pattern was indistinguishable from that observed in Wt-Tg501 mice infected with sheep-BSE

isolate. Moreover, an identical $PrP^{res}$ banding pattern was observed in Wt-Tg501 mice inoculated with Cattle-BSE isolate (Fig. 16C). By contrast, $PrP^{res}$ WB profile of the atypical BSE L, commonly characterized by a predominant monoglycosylated moiety and a smaller molecular size in the unglycosylated fragment than in classical cattle-BSE, was changed into a BSE-like WB profile upon passage in Wt-Tg501 mice (Fig. 16C).

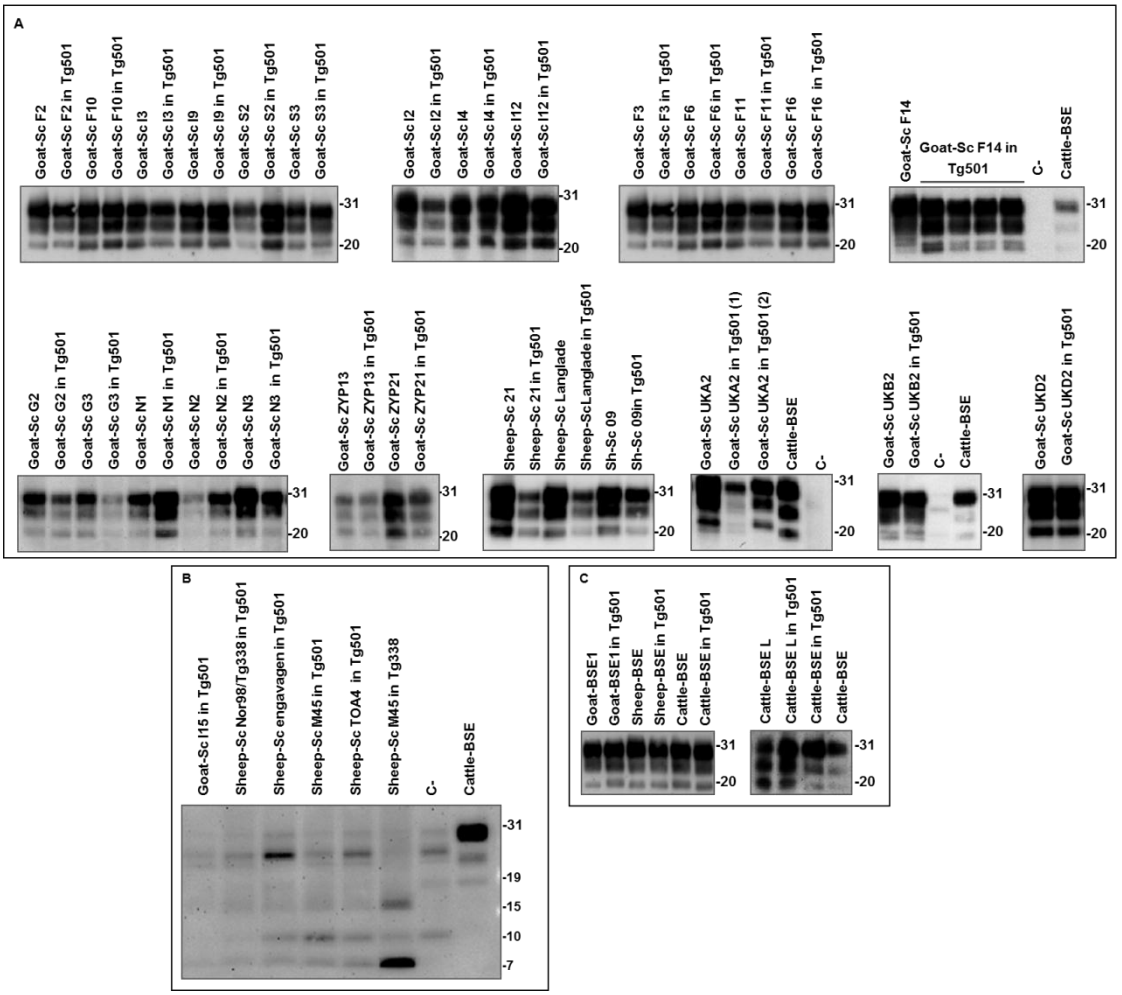


**Figure 16. $PrP^{res}$ of scrapie ad BSE isolates both before and after transmission in Wt-Tg501 mice** Immunoblots of brain $PrP^{res}$ from Wt-Tg501 mice infected with classical scrapie (A), atypical scrapie (B), and BSE, BSE L isolates (C). $PrP^{res}$ was detected with Sha31 mAb for classical scrapie-infected and BSE-infected mice and with 9A2 mAb for atypical scrapie-infected mice. Similar quantities of sample (10% brain homogenate) were loaded for adequate comparison. Molecular weights in kilodaltons are shown on the right-hand side of the blot. Goat-Sc UKA2 in Tg501 (1): some Goat-ScUKA2-infected Wt-Tg501 mice exhibited a 19K-21K double unglycosilated band in their brains. Goat-Sc UKA2 in Tg501 (2): some Goat-ScUKA2-infected Wt-Tg501 mice had a 21K single unglycosilated band. Sheep-Sc M45 passaged in VRQ-OvTg338 mice was used as positive control in atypical scrapie WBs. C-: non-inoculated Wt-Tg501 mouse brain.

### *1.2.2.3 Histopathological analysis*

Brains from all of the challenged Wt-Tg501 mice were routinely analyzed for the presence of $PrP^{Sc}$ deposition and neurodegenerative lesions by Paraffin-Embedded Tissue blotting (PET-blot) and hematoxilyn-eosin (HE) staining, respectively.

PET-blot analysis confirmed the presence of $PrP^{Sc}$ deposits in all the scrapie- or BSE-inoculated Wt-Tg501 mice (Fig. 17A-C). $PrP^{Sc}$ deposits were widely detected in the grey and white matter of all the studied coronal brain sections, including medulla oblongata, cerebellum, and brain stem. By contrast, no $PrP^{Sc}$ deposits or neurological lesions were detected in Wt-Tg501 mice inoculated with healthy goat brains. Only a few vacuolar changes were observed in areas of white matter, probably due to the advanced age of these animals (650 dpi).

Scrapie-infected mice had an extensive $PrP^{Sc}$ topographical distribution characterized by the presence of $PrP^{Sc}$ deposits throughout the dorsal medulla, cerebellum, thalamus, hippocampus, and septum (Fig. 17A and B). The cochlear and dorsal medial nuclei, raphe, dorsal colliculus, habenula, pyramidel tract, medial septal nucleus, and cuadoputamen were greatly affected. The cortex was also involved in the different studied brain sections and $PrP^{Sc}$ was mainly detected in its granular layer but also in its molecular layer. $PrP^{Sc}$ deposition was accompanied by high spongiform degeneration (Fig. 17C), above all at the level of white matter areas, as determined by HE staining.

Differences between scrapie isolates in the histopathological analysis were observed. Interestingly, these differences coincided with the three kinds of scrapie isolates identified in Wt-Tg501 mice in terms of their ST at second passage (Table 11): Italian isolates, non-Italian fast isolates, and non-Italian slow isolates. Non-Italian fast isolates (Goat-Sc F2 in Fig. 17) and non-Italian slow isolates (Goat-Sc F10 in Fig. 17) both had very similar $PrP^{Sc}$ deposition patterns (Fig. 17A and B) but clearly different spongiosis profiles (Fig. 17C). Among non-Italian fast isolates, those that had a doubled unglycosilated band (Goat-Sc F14, Goat-Sc UKA2 and Goat-Sc UKB2) showed no major differences in their histopathological alterations than the rest of the isolates of their group. Italian isolates showed very homogenous histopathological

outcomes which clearly differed from those of non-Italian isolates. In general, Italian isolates had lower levels of $PrP^{Sc}$ deposition with less involvement of the superior colliculus, dorsal raphe, lateral dorsal nucleus, and cortex (Fig. 17A and B).

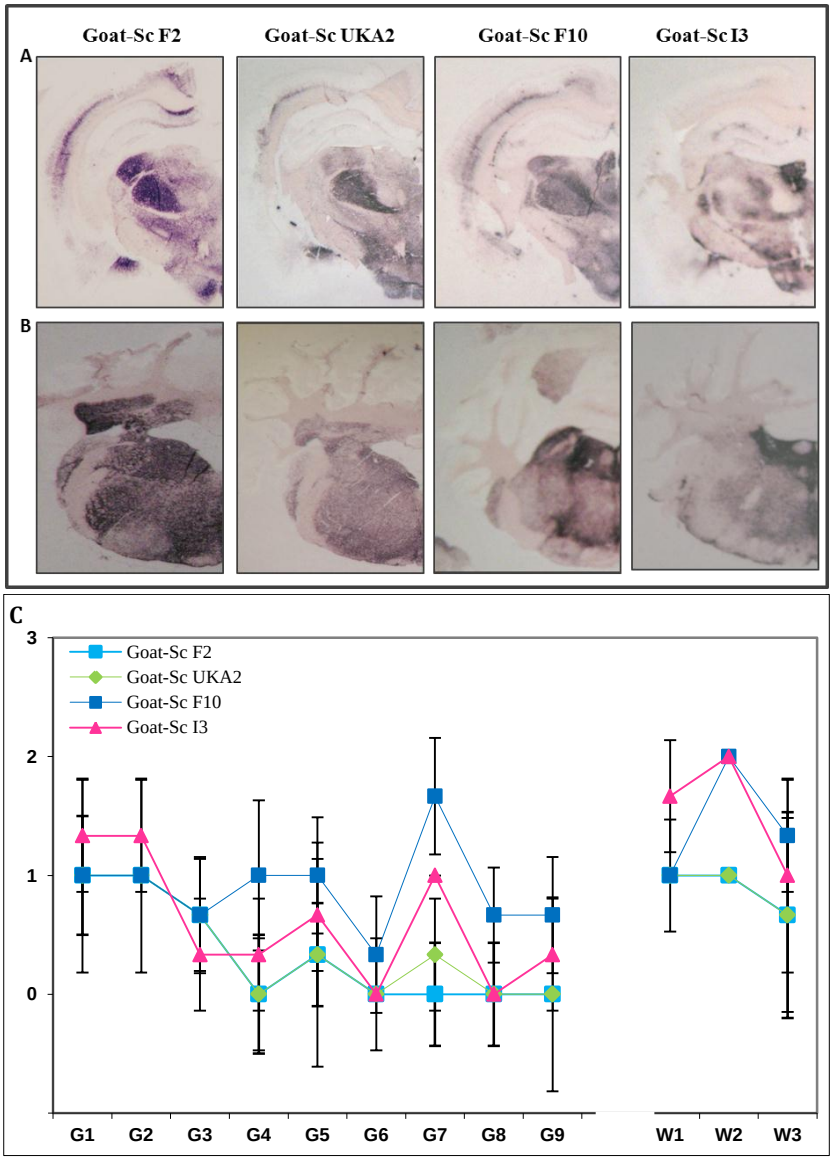


**Figure 17. $PrP^{Sc}$ distribution and lesion profile in brain of scrapie-inoculated Wt-Tg501 mice.** PET-blots of representative coronal sections at the level of the thalamus (A) and medulla (B) of Wt-Tg501 mice inoculated with Goat-Sc F2, Goat-Sc UKA2, Goat-Sc F10, and Goat-Sc I3 inocula. 20x magnification level was used. Vacuolar lesion profiles in brains (C) from Wt-Tg501 mice inoculated with Goat-Sc F2 (light blue squares, n = 3 animals), Goat-Sc UKA2 (light green rhombuses, n = 3 animals), Goat-Sc F10 (dark blue squares, n = 3 animals), and Goat-Sc I9 (pink triangles, n = 3 animals). Lesion scoring was carried out for nine areas of gray matter (G) and white matter (W) in mouse brains: dorsal medulla (G1), cerebellar cortex (G2), superior colliculus (G3), hypothalamus (G4), medial thalamus (G5), hippocampus (G6), septum (G7), medial cerebral cortex at the level of the thalamus (G8) and the septum (G9), cerebellum (W1), mesencephalic tegmentum (W2), and pyramidal tract (W3). Error bars indicate the standard error of the mean.

On the other hand, PET-blot studies in BSE-infected Wt-Tg501 mice revealed very similar $PrP^{Sc}$ distribution patterns (Fig. 18A) and lesion profiles (Fig. 18B) in animals inoculated either with classical cattle-BSE or goat-BSE isolates. $PrP^{Sc}$ deposits in these BSE-infected Wt-Tg501 mice had a more restricted distribution than in the scrapie-infected Wt-Tg501 mice and were mostly observed at the level of the cerebellum, ventral septum, and thalamus, with little involvement of the hippocampus (Fig. 18A). Spongiosis was in general less evident in BSE-infected mice than in scrapie-infected mice, and was mainly limited to the cerebellum, dorsal medulla, and white matter areas (Fig. 18B). Cattle-BSE L-infected mice differed from the rest of BSE-infected mice in their histopathological outcomes. $PrP^{Sc}$ deposits were more intense and abundant at the level of the thalamus and hippocampus (Fig. 18A) and spongiosis was noticeably more severe (Fig. 18B).

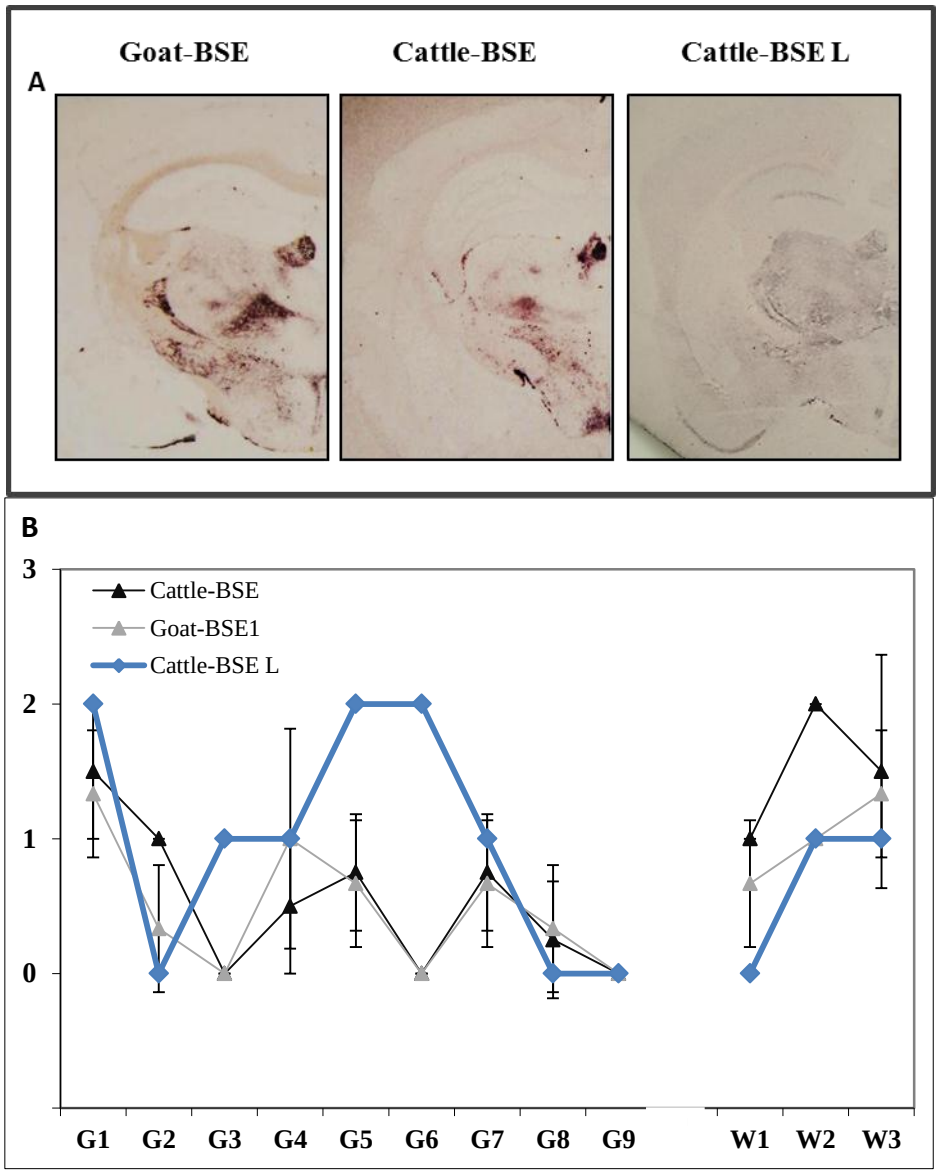


**Figure 18. $PrP^{Sc}$ distribution and lesion profile in brains of BSE-inoculated Wt-Tg501 mice.** PET-blots of representative coronal sections at the level of the thalamus (A) of Wt-Tg501 mice inoculated with Goat-BSE1, Cattle-BSE, and Cattle-BSE L inocula. 20x magnification was used. Vacuolar lesion profile (B) in brains from Wt-Tg501 mice inoculated with Cattle-BSE (black triangles, n = 6 animals), Goat-BSE1 (grey triangles, n = 3 animals), and Cattle-BSE L (blue rhombuses, n = 1 animal). Lesion scoring was undertaken for nine areas of gray matter (G) and white matter (W) in mouse brains: dorsal medulla (G1), cerebellar cortex (G2), superior colliculus (G3), hypothalamus (G4), medial thalamus (G5), hippocampus (G6), septum (G7), medial cerebral cortex at the level of the thalamus (G8) and the septum (G9), cerebellum (W1), mesencephalic tegmentum (W2), and pyramidal tract (W3). Error bars indicate the standard error of the mean.

## 1.3 Susceptibility of transgenic mice expressing different goat-PrP$^C$ polymorphic variants to prion infection

After confirming the suitability of the goat wt-PrP$^C$ Tg mouse line (Wt-Tg501) as a model for studying the susceptibility of goats to TSEs, transmission studies were carried out with the Tg mouse lines expressing different goat-PrP$^C$ polymorphic variants ($M_{142}$-, $K_{222}$-, $H_{154}$-, and $Q_{211}$-PrP$^C$ variants) in order to evaluate the individual role of each of these PrP$^C$ variants in resistance/susceptibility to prion infection. In this way, $K_{222}$-Tg516, $H_{154}$-Tg563, $Q_{211}$-Tg580, and $M_{142}$-Tg541 mouse lines were IC inoculated with a selection of scrapie and BSE isolates and their relative susceptibilities were assessed in comparison to those obtained in Wt-Tg501 mice.

A number of classical scrapie isolates from Spain, France, and Italy were selected from the wide range of scrapie isolates tested to maintain the geographical diversity. Nevertheless, only one atypical scrapie isolate was used for these transmission studies since all of the atypical scrapie isolates exhibited similar properties when transmitted to Wt-Tg501 mice (Tables 11). Sheep-Sc M45 was the atypical isolate selected because it produced the lowest ST and highest AR in Wt-Tg501 mice and harbors a sheep wild type PrP$^{Sc}$ primary sequence.

### *1.3.1 Prion transmission features in goat $K_{222}$-PrP$^C$ transgenic mice*

Of all the PrP$^C$ variants in goats, $K_{222}$ has yielded the most promising results in terms of resistance against TSEs. Its low susceptibility to classical scrapie infection has been documented in numerous epidemiological studies in several European countries (Acin et al., 2013; Acutis et al., 2006; Barillet et al., 2009; Bouzalas et al., 2010; Corbiere et al., 2013b; Fragkiadaki et al., 2011; Papasavva-Stylianou et al., 2011; Vaccari et al., 2006). However, this evidence has not yet been analyzed in detail by experimental inoculations. Taking into account these data, $K_{222}$-Tg516 mice were challenged with a broad panel of classical scrapie isolates to study the role of this polymorphism in susceptibility to scrapie

infection. In addition, $K_{222}$-Tg516 mice were inoculated with an atypical scrapie agent and a number of BSE isolates (including an atypical BSE L isolate).

$K_{222}$-Tg516 mice expressing the $K_{222}$-$PrP^C$ at around 1-fold the $PrP^C$ in the goat brain were resistant to the primary transmission of all the inoculated classical scrapie isolates (Table 12). These animals were sacrificed at the end of their lifespans (650 dpi) without any apparent clinical signs of the disease and their brains were scored as $PrP^{Sc}$ negative by WB and IHC (Andreoletti et al., 2000) analysis.

**Table 12.** Transmission of a panel of scrapie and BSE isolates to Wt-Tg501 and $K_{222}$-Tg516 mice

| | **Mean survival time in days ± SEM ($n/n_0$)[a]** | |
|---|---|---|
| **Isolate** | **Wt-Tg501 ($Q_{222}$/-) 1X[b]** | **$K_{222}$-Tg516 ($K_{222}$/-) 1X** |
| **Goat-Sc F2** | 250±36 (4/4) | >650 (0/7) |
| **Goat-Sc F10** | 465±19(7/7) | >650 (0/5) |
| **Goat-Sc I3** | >650 (5/5) | >650 (0/5) |
| **Goat-Sc I9** | 600±43 (5/5) | >650 (0/5) |
| **Goat-Sc S2** | 449±62 (9/9) | >650 (0/6) |
| **Goat-Sc S3** | 298±22 (6/6) | >650 (0/6) |
| **Sheep-Sc 21** | 500±23 (4/4) | >650 (0/6) |
| **Sheep-Sc Langlade** | >650 (3/3) | >650 (0/5) |
| **Sheep-Sc M45[c]** | 453±12 (5/5) | 416±12 (4/4) |
| **Goat-BSE1** | 497±31 (5/5) | 519±42 (5/5) |
| **Goat-BSE2** | 484±34 (7/7) | 478±31 (4/4) |
| **Sheep-BSE** | 485±62 (7/7) | 418±11 (4/4) |
| **Cattle-BSE** | 583±57 (6/6) | >650 (0/6) |
| **Cattle-BSE/Tg516[d]** | NA | >650 (0/6) |
| **Cattle-BSE/Tg501[e]** | 326±26 (6/6) | 434±30 (5/5) |
| **Cattle-BSE L** | 618±41 (4/4) | >650 (0/6) |
| **Healthy goat brain** | >650 (0/6) | >650 (0/6) |

[a] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

**[b]** $PrP^C$ level of expression in the brain compared to $PrP^C$ in goat and sheep brains as assessed by Western blot analysis (see Fig. 13A and E).

[c] Atypical scrapie isolate inoculated in homozygous Wt-Tg501 (2X) and $K_{222}$-Tg516 mice (2X).

[d] Pool of $K_{222}$-Tg516 mice inoculated with Cattle-BSE isolate.

[e] Pool of terminally ill Wt-Tg501 mice inoculated with Cattle-BSE isolate.

NA: Not analyzed

Conversely, $K_{222}$-Tg516 mice succumbed to the IC inoculation of the atypical scrapie isolate (Table 12). These mice had 100% AR and similar ST to the Wt-Tg501 mice inoculated with the same isolate.

The $K_{222}$-Tg516 mouse line was fully susceptible (100% AR) to goat and sheep BSE isolates and had similar mean ST (in the range 400–500 dpi) to Wt-Tg501 mice (Table 12). Nevertheless, $K_{222}$-Tg516 mice did not succumb to either classical Cattle-BSE or to atypical Cattle-BSE L. These mice were sacrificed at the end of their lifespans (650 dpi) without any apparent clinical signs or $PrP^{res}$ in the brain. Moreover, the resistance in $K_{222}$-Tg516 mice to classical cattle BSE was even maintained after second passage (Cattle-BSE/Tg516, Table 12). Surprisingly, $K_{222}$-Tg516 mice were fully susceptible to the cattle BSE agent after passage in Wt-Tg501 mice (Cattle-BSE/Tg501), with a mean ST of 434±30 dpi.

None of the $K_{222}$-Tg516 mice inoculated with healthy goat brain showed any evidence of neurological disease until the end of their lifespan (Table 12) and their brains were scored as $PrP^{Sc}$ negative by WB, PET-blot and IHC analysis, which discards any spontaneous development of a prion disease in these mice.

Western blot analysis of brain $PrP^{res}$ in goat- and sheep-BSE-infected $K_{222}$-Tg516 mice showed the typical $PrP^{BSE}$ banding pattern previously described for Wt-Tg501 mice inoculated with goat, sheep, or cattle-BSE agents (Fig. 19A). Likewise, the $PrP^{res}$ WB profile of the atypical scrapie isolate was also maintained after passage in $K_{222}$-Tg516 mice (Fig. 19B).

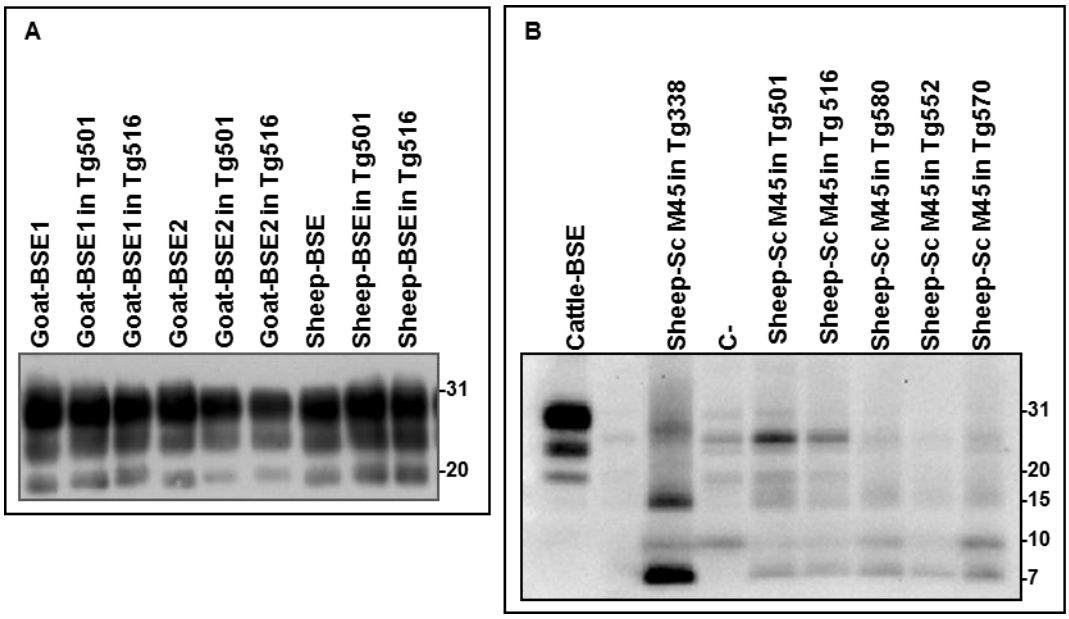


**Figure 19. $PrP^{res}$ of scrapie ad BSE isolates both before and after transmission in either Wt-Tg501 mice or $K_{222}$-Tg516**. (A) Immunoblots of brain $PrP^{res}$ from Wt-Tg501 and $K_{222}$-Tg516 mice infected with goat- and sheep-BSE isolates. (B) Immunoblots of brain $PrP^{res}$ from Wt-Tg501, $K_{222}$-Tg516, $Q_{211}$-Tg580, $R_{171}$-Tg552 and $K_{176}$-Tg570 mice infected with atypical scrapie isolate. $PrP^{res}$ was detected with Sha31 mAb for BSE-infected mice and with 9A2 mAb for atypical scrapie-infected mice. Similar quantities of sample (10% brain homogenate) were loaded for adequate comparison. Molecular weights in kilodaltons are shown on the right-hand side of the blot. Sheep-Sc M45 passaged in VRQ-OvTg338 mice was used as positive control in atypical scrapie WBs. C-: non-inoculated Wt-Tg501 mouse brain.

The absence of $PrP^{Sc}$ in the brains of classical scrapie-challenged $K_{222}$-Tg516 mice was confirmed by PET-blot (Fig. 20A). These animals exhibited some vacuolation, mainly at the level of the dorsal medulla and mesencephalic tegmentum. However, these alterations were also observed in the same areas in old Wt-Tg501 and $K_{222}$-Tg516 mice inoculated with healthy goat brain, probably due to the advanced age of these animals.

$PrP^{Sc}$ deposits or spongiform changes were not detected in the brains of Cattle-BSE-infected $K_{222}$-Tg516 mice (Fig. 20A). These $K_{222}$-Tg516 mice exhibited some spongiosis, mainly at the level of the dorsal medulla and the mesencephalic tegmentum, that coincided with the vacuolation observed in old Wt-Tg501 and $K_{222}$-Tg516 mice inoculated with healthy goat brain, which again indicates that the old age of the animals was the cause of these lesions. However, when $K_{222}$-Tg516 mice were infected with goat BSE isolates, they exhibited $PrP^{res}$ deposits

and vacuolation that were very similar to those observed in Wt-Tg501 mice infected with either cattle or goat BSE isolates (Fig. 20 A and B).

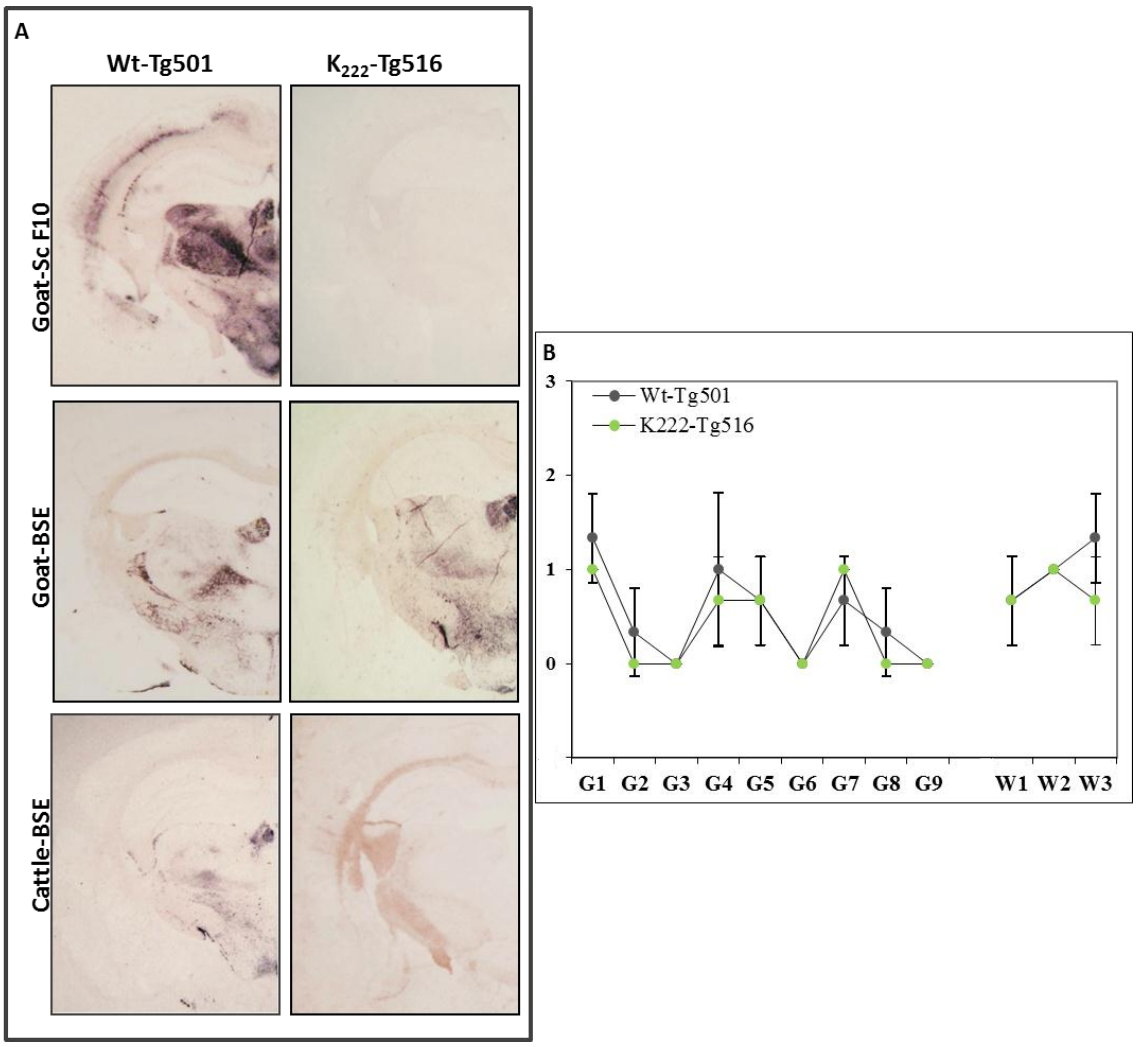


**Figure 20. $PrP^{Sc}$ distribution and lesion profile in brain of scrapie and BSE-inoculated $K_{222}$-Tg516 mice in comparison with Wt-Tg501 mice.** PET-blots of representative coronal sections (A) at the level of the thalamus of Wt-Tg501and $K_{222}$-Tg516 mice inoculated with Goat-Sc F10, Goat-BSE, and Cattle-BSE. 20x magnification level was used. Vacuolar lesion profile (B) in brains from $K_{222}$-Tg516 (green circles, n = 4 animals) mice and Wt-Tg501 (grey circles, n = 3 animals) mice inoculated with Goat-BSE1. Lesion scoring was carried out for nine areas of gray matter (G) and white matter (W) in mouse brains: dorsal medulla (G1), cerebellar cortex (G2), superior colliculus (G3), hypothalamus (G4), medial thalamus (G5), hippocampus (G6), septum (G7), medial cerebral cortex at the level of the thalamus (G8) and the septum (G9), cerebellum (W1), mesencephalic tegmentum (W2), and pyramidal tract (W3). Error bars indicate SE.

### *1.3.2 Prion transmission features in goat $Q_{211}$-$PrP^C$ transgenic mice*

$R/Q_{211}$ polymorphisms have been epidemiologically linked to low susceptibility to classical scrapie (Barillet et al., 2009; Bouzalas et al., 2010); however, little is known about their role in BSE infection. In these experiments, $Q_{211}$-Tg580 mice were bred to homozygosis in order to obtain similar brain $PrP^C$ levels of expression than Wt-Tg501 mice (1X). Homozygous $Q_{211}$-Tg580 mice were challenged with a variety of scrapie and BSE isolates.

Homozygous $Q_{211}$-Tg580 mice expressing the $Q_{211}$-$PrP^C$ at around 1-fold the $PrP^C$ in the goat brain did not succumb to the primary transmission of any of the classical scrapie isolates that were inoculated (Table 13). These mice were euthanized without showing any apparent neurological sign of prion disease or $PrP^{Sc}$ in their brains after analysis by WB or PET-blot. However, $Q_{211}$-Tg580 mice did succumb to the infection with atypical scrapie isolate (Table 13). These animals were euthanized showing no sign of prion disease but were scored $PrP^{res}$ positive in their brains by WB analysis (Fig. 19). Brain $PrP^{res}$ WB profile of infected $Q_{211}$-Tg580 mice was very similar to that previously observed for Wt-Tg501 mice (Fig. 19B).

**Table 13.** Transmission of a panel of scrapie and BSE isolates to Wt-Tg501 and $Q_{211}$-Tg580 mice

| | **Mean survival time in days ± SEM ($n/n_0$)[a]** | |
|---|---|---|
| **Isolate** | **Wt-Tg501 ($Q_{211}$/-) 1X[b]** | **$Q_{211}$-Tg580 ($Q_{211}$/ $Q_{211}$) 1X** |
| **Goat-Sc F2** | 250±36 (4/4) | >650 (0/7) |
| **Goat-Sc F10** | 465±19(7/7) | >650 (0/5) |
| **Goat-Sc I3** | >650 (5/5) | >650 (0/5) |
| **Sheep-Sc Langlade** | >650 (3/3) | >650 (0/5) |
| **Sheep-Sc M45[c]** | 453±12 (5/5) | >650 (4/4) |
| **Goat-BSE2** | 484±34 (7/7) | >550 (ongoing) |
| **Sheep-BSE** | 485±62 (7/7) | >550 (ongoing) |
| **Cattle-BSE** | 583±57 (6/6) | >550 (ongoing) |
| **Healthy goat brain** | >650 (0/6) | >650 (0/6) |

[a] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

[b] $PrP^C$ level of expression in the brain compared to $PrP^C$ in goat and sheep brains as assessed by Western blot analysis (Fig. 13A and D).

[c] Atypical scrapie isolate Sheep-Sc M45 inoculated in homozygous Wt-Tg501 (2X) and $Q_{211}$-Tg580 mice (1X).

$Q_{211}$-Tg580 mice have not yet succumbed to any of the BSE isolates inoculated. These mice surpass the ST previously observed in BSE-infected Wt-Tg501 mice. Again, no prion disease was evidenced in $Q_{211}$-Tg580 mice inoculated with healthy goat brain (Table 13). WB and PET-blot analysis discarded the presence of $PrP^{Sc}$ in their brains.

### *1.3.3 Prion transmission features in goat $H_{154}$-$PrP^C$ transgenic mice*

$R/H_{154}$ polymorphism have also been associated to low susceptibility to classical scrapie in natural outbreaks (Barillet et al., 2009; Billinis et al., 2002), even though this polymorphism has also been reported to be a risk factor for atypical scrapie (Benestad et al., 2008; Colussi et al., 2008). In these experiments, $H_{154}$-Tg563 mice were challenged with some classical scrapie and BSE isolates. The lower $PrP^C$ levels of expression in the brain of this mouse line compared to goat Wt-Tg501 mice (0.25-fold the $PrP^C$ in the brain of Wt-Tg501 mice, respectively) were taken into account when performing the comparative susceptibility of $H_{154}$-$PrP^C$ variant to the prion infection.

$H_{154}$-Tg563 mice did not succumb to the primary transmission of any classical scrapie isolate except for Goat-Sc F2 (Table 14). $H_{154}$-Tg563 mice were 80% susceptible to this scrapie isolate and had much longer ST than Wt-Tg501 mice. When inoculated with BSE, $H_{154}$-Tg563 mice only succumbed to Goat-BSE2 isolate that had very long ST (582 dpi) and very low AR (20%).

**Table 14.** Transmission of a panel of scrapie and BSE isolates to Wt-Tg501 and $H_{154}$-Tg563 mice

| Isolate | Mean survival time in days ± SEM ($n/n_0$)[a] | |
|---|---|---|
| | **Wt-Tg501 ($H_{154}Q_{211}$/-) 1X[b]** | **$H_{154}$-Tg563 ($H_{154}$/-) 0.25X** |
| **Goat-Sc F2** | 250±36 (4/4) | 650±9 (5/6) |
| **Goat-Sc F10** | 465±19(5/5) | >650 (0/5) |
| **Goat-Sc I3** | >650 (5/5) | >650 (0/6) |
| **Goat-Sc I9** | 600±43 (5/5) | >650 (0/6) |
| **Goat-Sc S2** | 449±62 (9/9) | >650 (0/6) |
| **Goat-BSE1** | 497±31 (5/5) | >650 (0/6) |
| **Goat-BSE2** | 484±34 (7/7) | 582 (1/5) |
| **Cattle-BSE** | 501±35 (6/6) | >650 (0/6) |
| **Healthy goat brain** | >650 (0/6) | >650 (0/6) |

[a] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

[b] $PrP^C$ level of expression in the brain compared to $PrP^C$ in goat and sheep brains as assessed by Western blot analysis (Fig. 13A and C).

$H_{154}$-Tg563 mice inoculated with healthy goat brain showed any sign of neurological disease (Table 14). Neither $PrP^{res}$ nor histopathological alterations were observed in the brains of these animals.

Attending to the $PrP^{res}$ WB glycoprofile, no differences were observed between Wt-Tg501 and $H_{154}$-Tg563 infected mice (Fig. 21).

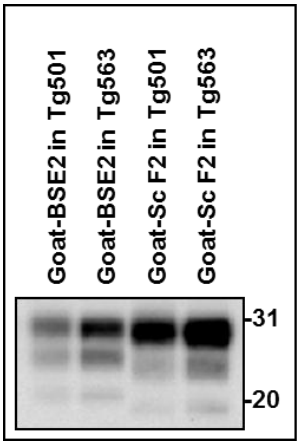


**Figure 21. $PrP^{res}$ of Goat-BSE2 and Goat-ScF2 after transmission in Wt-Tg501 and $H_{154}$-Tg563 mice.** Immunoblots of brain $PrP^{res}$ from Wt-Tg501 and $H_{154}$-Tg563 mice infected with Goat-BSE2 and Goat-Sc F2 isolates detected with Sha31 mAb. Similar quantities of sample (10% brain homogenate) were loaded for adequate comparison. Molecular weights in kilodaltons are shown on the right-hand side of the blot.

Finally, $PrP^{Sc}$ deposition and spongiform changes were only detected in the brains of $H_{154}$-Tg563 mice infected with Goat-Sc F2 and Goat-BSE2. No major differences were observed in the histopathological outcomes of $H_{154}$-Tg563 mice compared to Wt-Tg501 mice (data not shown).

### *1.3.4 Prion transmission features in goat $M_{142}$-$PrP^C$ transgenic mice*

$I/M_{142}$ polymorphism has been associated with decreased susceptibility to BSE and scrapie in experimentally inoculated goats (Goldmann et al., 1996). Moreover, several field studies have linked this $PrP^C$ variant to low susceptibility to classical scrapie (Barillet et al., 2009; Goldmann et al., 2011; Gonzalez et al., 2010). Nevertheless, the role of $M_{142}$-$PrP^C$ variant in the resistance/susceptibility of goats to atypical scrapie infection has not yet been assessed. Therefore, in order to clarify the effect of this goat $PrP^C$ polymorphism on prion infection, Tg mice expressing the $M_{142}$-$PrP^C$ variant were IC inoculated with a panel of scrapie and BSE isolates and their transmission features were compared to those of goat Wt-Tg501 mice.

$M_{142}$-Tg541 mice expressing the $M_{142}$-$PrP^C$ at around 1-fold the $PrP^C$ in the goat brain were fully susceptible to the primary transmission of most of the classical scrapie isolates tested here, albeit with significantly longer ST than those observed in Wt-Tg501 (Table 15). Only two of the isolates (Goat-Sc I3 and Goat-Sc I9) were unable to transmit in this mouse model, probably due to an increase in ST that was already very long in Wt-Tg501 mice. These $M_{142}$-Tg541 mice were scored as $PrP^{res}$ negative in their brains and had no neurological lesions or $PrP^{Sc}$ deposits (Fig. 22).

More interestingly, $M_{142}$-Tg541 mice were resistant to the primary transmission of the atypical scrapie isolate Sheep-Sc M45 (Table 15). None of these mice showed any sign of neurological disease until the end of their lifespans and no $PrP^{res}$ was detected in their brains by WB analysis.

In terms of BSE transmissions, $M_{142}$-Tg541 mice were 100% susceptible to all the inoculated BSE isolates regardless of the PrP primary sequence of the isolate

(cattle, sheep, or goat), as previously observed in Wt-Tg501 mice (Table 15). Once again, ST were slightly longer than those obtained in Wt-Tg501 mice (except for the Sheep-BSE isolate); however, no statistically significant differences were detected in these cases (Table 15).

**Table 15.** Transmission of a panel of scrapie and BSE isolates to Wt Tg501 and $M_{142}$-Tg541 mice

| Isolate | Mean survival time in days ± SEM ($n/n_0$)[a] | | *P value*[c] |
|---|---|---|---|
| | **Wt-Tg501 ($I_{142}$/-) 1X[b]** | **$M_{142}$-Tg541 ($M_{142}$/-) 1X** | |
| **Goat-Sc F2** | 250±36 (4/4) | 423±48 (7/7) | *0.01073* |
| **Goat-Sc F10** | 465±19(5/5) | 542±26 (5/5) | *0.01219* |
| **Goat-Sc I3** | >650 (5/5) | >650 (0/5) | - |
| **Goat-Sc I9** | 600±43 (5/5) | >650 (0/5) | - |
| **Goat-Sc S2** | 449±62 (9/9) | 543±55(6/6) | *0.04883* |
| **Goat-Sc S3** | 298±22 (6/6) | 439±65 (5/5) | *0.008113* |
| **Sheep-Sc Langlade** | >650 (3/3) | 650±9 (3/4) | *0.0489* |
| **Sheep-Sc M45[d]** | 453±12 (5/5) | >650 (0/6) | - |
| **Goat-BSE1** | 497±31 (5/5) | 521±46(5/5) | *0.4034* |
| **Goat-BSE2** | 484±34 (7/7) | 537±65(6/6) | *0.2298* |
| **Sheep-BSE** | 485±62 (7/7) | 423±36 (3/3) | *0.1715* |
| **Cattle-BSE** | 501±35 (6/6) | 540±24 (4/4) | *0.5959* |
| **Healthy goat brain** | >650 (0/6) | >650 (0/6) | - |

[a] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

**[b]** $PrP^C$ level of expression in the brain compared to $PrP^C$ in goat and sheep brains as assessed by Western blot analyses (see Fig. 13A and B).

**[c]** A non-parametric Mann-Whitney-U test was used to establish statistically significant differences in survival times between Wt-Tg501 and $M_{142}$-Tg541 mouse lines inoculated during the different scrapie and BSE challenges. A difference of $P<0.05$ was considered to be significant.

[d] Atypical scrapie isolate was inoculated in homozygous Wt-Tg501 (2X) and $M_{142}$-Tg541 mice (2X).

Finally, when the $M_{142}$-Tg541 mice inoculated with the healthy goat brain were euthanized at the end of their lifespan (650 dpi), they had no clinical signs of disease or $PrP^{res}$ in their brains, thereby ruling out the spontaneous development of disease.

Brain $PrP^{res}$ WB glycoprofile of infected $M_{142}$-Tg541 mice were undistinguishable from those of infected Wt-Tg501 independently of the inoculated isolate (**F**ig. 22).

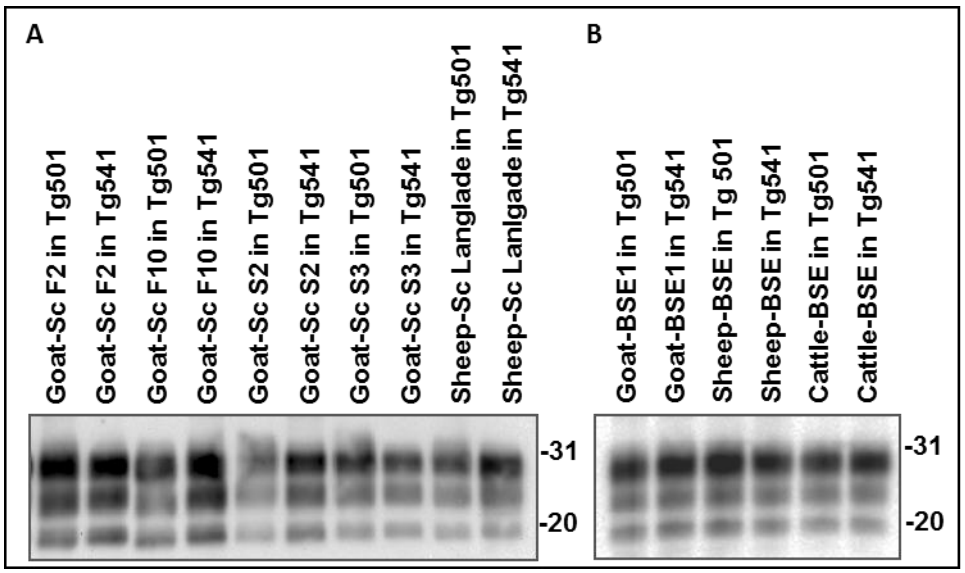


**Figure 22. $PrP^{res}$ of classical scrapie and BSE isolates after transmission in Wt-Tg501 and $M_{142}$-Tg541 mice.** Immunoblots of brain $PrP^{res}$ from Wt-Tg501 and $M_{142}$-Tg541 mice infected with classical scrapie (A) and BSE (B) isolates detected with Sha31 mAb. Similar quantities of sample (10% brain homogenate) were loaded for adequate comparison. Molecular weights in kilodaltons are shown on the right-hand side of the blot.

Histopathological studies confirmed the presence of $PrP^{Sc}$ deposition and spongiform changes in the brains of all the $M_{142}$-Tg541 mice infected with either BSE or classical scrapie isolates, except for those inoculated with Goat-Sc I3 (Fig. 23A) or healthy goat brain. $PrP^{res}$ deposition (Fig. 23A) and vacuolation profiles (Fig. 23B) in $M_{142}$-Tg541 animals mostly overlapped with those previously determined in Wt-Tg501 mice. However, Goat-Sc F2 and Goat-Sc S2 produced different vacuolation profiles in $M_{142}$-Tg541 animals from those in Wt-Tg501 mice, even though the distribution of $PrP^{Sc}$ deposits was very similar in both mouse lines (Fig. 23).

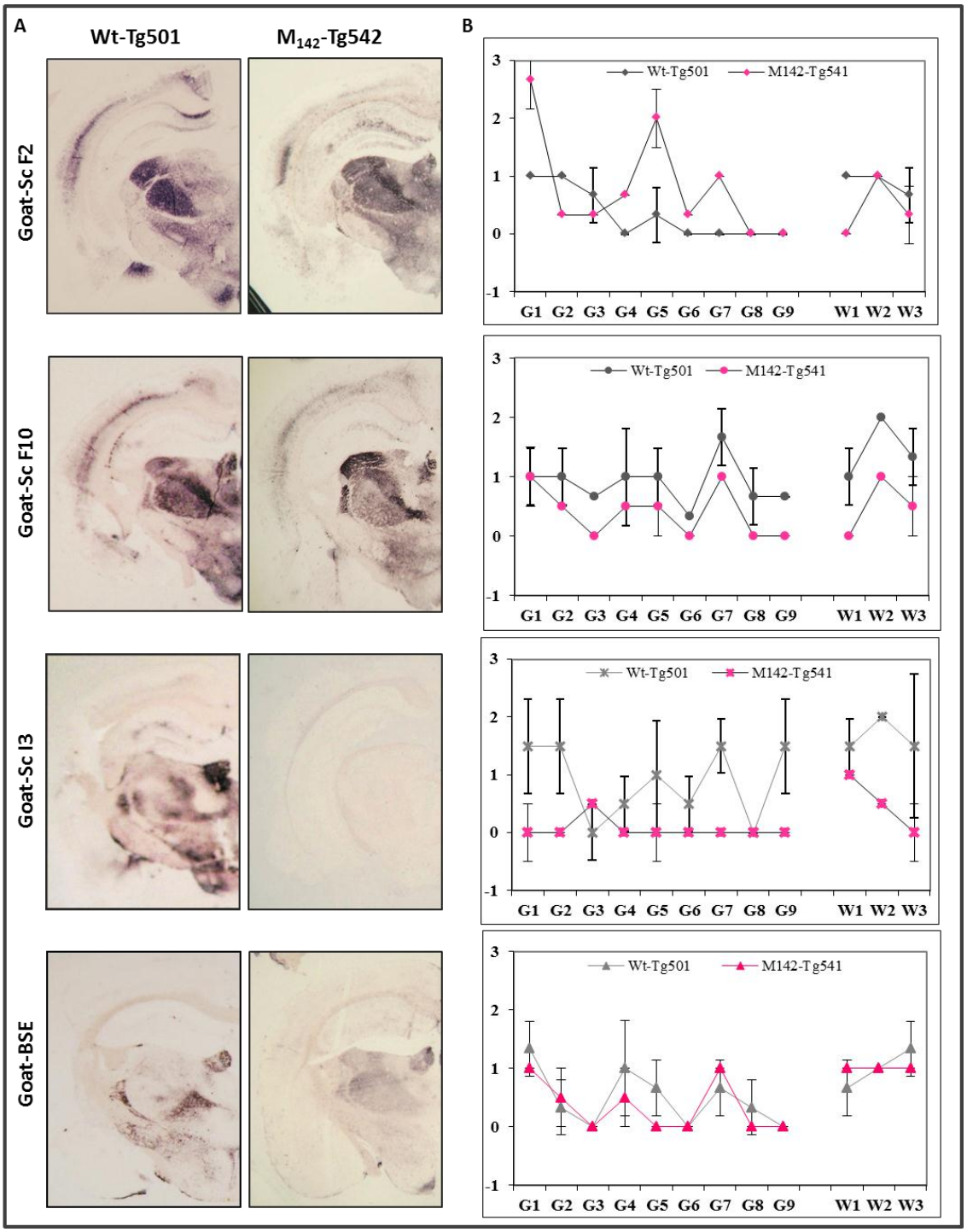


**Figure 23. PrP$^{Sc}$ distribution and lesion profile in brains of scrapie- and BSE-inoculated $M_{142}$-Tg541 mice.** PET blots of representative coronal sections (A) at the level of thalamus of Wt-Tg501 and $M_{142}$-Tg541 mice inoculated with Goat-Sc F2, Goat-Sc F10, Goat-Sc I3, and Goat-BSE. 20x magnification level was used. Vacuolar lesion profile (B) in brains from both Wt-Tg501 and $M_{142}$-Tg541 mice inoculated with Goat-Sc F2 (rhombuses, n = 4 animals), Goat-Sc F10 (circles, n = 4 animals), Goat-Sc I3 (crosses, n = 4 animals), and Goat-BSE (triangles, n = 5 animals). Lesion scoring was carried out as previously described for Wt-Tg501 mice (see section 1.2.2.3). Error bars indicate the standard error of the mean.

## 1.4 Susceptibility of transgenic mice expressing different sheep-PrP$^C$ polymorphic variants

### *1.4.1 Prion transmission studies in $R_{171}$-PrP$^C$ transgenic mice*

Due to its resistance to classical scrapie and BSE agents, $R_{171}$-PrP$^C$ variant is widely used in breeding programs aimed at controlling and eradicating scrapie in sheep flocks. However, $A_{136}R_{154}R_{171}$/ARR sheep succumb to cattle and sheep BSE infection after intracerebral challenges (Gonzalez et al., 2005) and natural cases of scrapie have been reported in these sheep from Germany and France (Groschup et al., 2007), and Japan (Ikeda et al., 1995). More recently, goats have been reported to harbor the Q/$R_{171}$ polymorphism whose resistance to classical scrapie infection has been compared to that provided by the sheep $A_{136}R_{154}R_{171}$ genotype (Bouzalas et al., 2010). In the present study, we analyzed the susceptibility of $R_{171}$-PrP$^C$ variant to prion infection and focused on the characterization of its assumed resistance to classical scrapie.

$R_{171}$-Tg552 mice expressing the $R_{171}$-PrP$^C$ variant at 1-fold the PrP$^C$ in sheep brains were resistant to the primary inoculation of all the classical scrapie inoculated isolates (Table 16). All of these $R_{171}$-Tg552 mice were euthanized at 650 dpi showing neither clinical signs nor PrP$^{Sc}$ in their brains by WB and PET-blot analysis. On the other hand, none of the BSE-inoculated $R_{171}$-Tg552 mice have yet succumbed to the prion disease and their ST now surpass those of their counterparts Wt-Tg501 mice (Table 16).

Interestingly, $R_{171}$-Tg552 mice were susceptible to atypical scrapie isolate (Table 16). Although mice showed no sign of the neurological disease until they were euthanized, PrP$^{res}$ was detected in their brains by WB analysis (Fig. 19). PrP$^{res}$ WB profile in the brain of these animals showed no major differences to that previously observed for Wt-Tg501 mice (Fig. 19B).

Finally, $R_{171}$-Tg552 mice did not develop any neurological sign after inoculated with healthy goat brain (Table 16).

**Table 16.** Transmission of a panel of scrapie and BSE isolates to Wt-Tg501 and $R_{171}$-Tg552 mice

| Isolate | Mean survival time in days ± SEM ($n/n_0$)[a] | |
|---|---|---|
| | **Wt-Tg501 ($Q_{171}$/-) 1X[b]** | **$R_{171}$-Tg552 ($R_{171}$/-) 1X** |
| **Goat-Sc F2** | 250±36 (4/4) | >650 (0/6) |
| **Goat-Sc F10** | 465±19(5/5) | >650 (0/6) |
| **Goat-Sc I3** | >650 (5/5) | >650 (0/7) |
| **Goat-Sc I9** | 600±43 (5/5) | >650 (0/5) |
| **Goat-Sc S2** | 449±62 (9/9) | >650 (0/5) |
| **Goat-Sc S3** | 298±22 (6/6) | >650 (0/6) |
| **Sheep-Sc 21** | 500±23 (4/4) | >650 (0/5) |
| **Sheep-ScLanglade** | >650 (3/3) | >650 (0/6) |
| **Sheep-Sc M45[c]** | 453±12 (5/5) | >650 (5/5) |
| **Goat-BSE2** | 484±34 (7/7) | >550 (ongoing) |
| **Sheep-BSE** | 485±62 (7/7) | >550 (ongoing) |
| **Cattle-BSE** | 501±35 (6/6) | >550 (ongoing) |
| **Healthy goat brain** | >650 (0/6) | >650 (0/6) |

[a] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

[b] $PrP^C$ level of expression in the brain compared to $PrP^C$ in goat and sheep brains as assessed by Western blot analysis (Fig. 13A,C and D).

[c] Atypical scrapie isolate Sheep-Sc M45 inoculated in homozygous Wt-Tg501 (2X) and $R_{171}$-Tg552 mice (2X).

### *1.4.2 Prion transmission studies in $K_{176}$-$PrP^C$ transgenic mice*

$K_{176}$ variant of the sheep $PrP^C$ was associated with a protective effect against the IC infection with classical scrapie and BSE (Vaccari et al., 2009b). This allele has only once been observed in a diseased sheep originating from a scrapie-affected herd (Vaccari et al., 2007); although the low frequency of this allele in healthy animals prevents from determining any significant protective effect (Bossers et al., 1996; Thorgeirsdottir et al., 1999; Vaccari et al., 2001). Thus, hemizygous $K_{176}$-Tg570 mice expressing 2-fold the $PrP^C$ in a sheep brain were challenged with scrapie and BSE isolates and their transmission features were compared to those of homozygous Wt-Tg501 (likewise 2-fold the $PrP^C$ in a sheep brain).

$K_{176}$-Tg570 mice were resistant to the primary transmission of all the classical scrapie isolates inoculated (Table 17). These animals were euthanized at the end of their lifespan and no $PrP^{res}$ or histopathological alteration was detected in their brains by WB or PET-blot analysis.

**Table 17.** Transmission of a panel scrapie and BSE isolates to Wt-Tg501 and $K_{176}$-Tg570 mice

| Isolate | Mean survival time in days ± SEM ($n/n_0$)[a] | |
|---|---|---|
| | Wt-Tg501 ($N_{176}$/ $N_{176}$) 2X[b] | $K_{176}$-Tg570 ($K_{176}$/-) 2X |
| **Goat-Sc F2** | 259±28 (4/4) | >650 (0/5) |
| **Goat-Sc F10** | 449±19 (5/5) | >650 (0/6) |
| **Goat-Sc I3** | >650 (5/5) | >650 (0/6) |
| **Goat-Sc I9** | 578±25 (5/5) | >650 (0/6) |
| **Goat-Sc S2** | 228±15 (6/6) | >650 (0/5) |
| **Goat-Sc S3** | 221±16 (6/6) | >650 (0/7) |
| **Sheep-Sc 21** | 194±5 (6/6) | >650 (0/6) |
| **Sheep-Sc Langlade** | >650 (3/3) | >630 (0/2) |
| **Sheep-Sc M45[c]** | 453±12 (5/5) | 362±26 (7/7) |
| **Goat-BSE1** | 366±24 (5/5) | 19K-BSE |
| **Sheep-BSE** | 405±37 (7/7) | >650 (0/5) |
| **Ca-BSE** | 357±9 (6/6) | >650 (0/5) |
| **Healthy goat brain** | >650 (0/6) | >650 (0/6) |

[a] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

[b] $PrP^C$ level of expression in the brain compared to $PrP^C$ in goat and sheep brains as assessed by Western blot analysis (Fig. 13A and G).

[c] Atypical scrapie isolate Sheep-Sc M45 inoculated in homozygous Wt-Tg501 (2X) and hemizgygous $K_{176}$-Tg570 mice (2X).

Intriguingly, $K_{176}$-Tg570 mice were 100% susceptible to the inoculation with atypical scrapie Sheep-Sc M45 (Table 17) and had statistically significant ($P$<0.01041) shorter ST than Wt-Tg501 mice (Mann-Whitney-U test). No changes in the $PrP^{res}$ profile of atypical scrapie isolates were detected after passage in these $K_{176}$-Tg570 mice (Fig. 19B).

$K_{176}$-Tg570 mice were resistant to BSE infection regardless of its $PrP^{Sc}$ primary sequence (cattle, sheep, or goat) (Table 17). They were euthanized at the end of their lifespans without showing any apparent clinical signs of prion disease and no $PrP^{res}$ was detected in their brains by WB and PET-blot analysis

Finally, none of the $K_{176}$-Tg570 mice inoculated with healthy goat brain developed any neurological disease.

### 1.5 Protective effect of $K_{222}$-$PrP^C$ on prion replication of the goat WT allele in heterozygous Q/$K_{222}$ transgenic mice

Homozygous $K_{222}$-Tg516 mice were crossbred with homozygous Wt-Tg501 mice to generate heterozygous (Q/$K_{222}$) mice. These animals were intracerebrally inoculated with the same panel of TSE isolates previously used for Wt-Tg501 and$K_{222}$-Tg516 mice challenges (Table 12) and their susceptibilities were compared to those of Wt-Tg501 and $K_{222}$-Tg516 Tg mouse lines.

Heterozygous Q/$K_{222}$ animals were resistant to the primary transmission of Goat-Sc I3 and Goat-Sc S2 isolates (Table 18). These animals were sacrificed at 650 dpi without showing any clinical signs of neurological disease; no detectable $PrP^{res}$ was found in their brains by either WB, IHC, or PET-blot analysis. However, Q/$K_{222}$ mice succumbed to the infection with the rest of scrapie isolates inoculated and had reduced AR and/or much longer ST than those observed in Wt-Tg501 mice. The Q/$K_{222}$ mice challenged with Goat-Sc F10 and Sheep-Sc Langlade isolates were sacrificed at 650 dpi without exhibiting any apparent clinical signs but with $PrP^{res}$ present in their brains (Table 18).

**Table 18.** Transmission of a panel of scrapie and BSE isolates to Wt-Tg501, $K_{222}$-Tg516, and heterozygous Q/$K_{222}$ mice

| | **Mean survival time in days ± SEM ($n/n_0$)[a]** | | |
|---|---|---|---|
| **Isolate** | **Wt-Tg501 ($Q_{222}$/-)** | **$K_{222}$-Tg516 ($K_{222}$/-)** | **Tg501 x Tg516 (Q/$K_{222}$)[b]** |
| **Goat-Sc F2** | 250±36 (4/4) | >650 (0/7) | 630±26 (5/6) |
| **Goat-Sc F10** | 465±19(7/7) | >650 (0/5) | >650 (6/6) |
| **Goat-Sc I3** | >650 (5/5) | >650 (0/5) | >650 (0/6) |
| **Goat-Sc I9** | 600±43 (5/5) | >650 (0/5) | >580 (ongoing) |
| **Goat-Sc S2** | 449±62 (9/9) | >650(0/6) | >650 (0/6) |
| **Goat-Sc S3** | 298±22 (6/6) | >650 (0/6) | >650 (2/6) |
| **Sheep-Sc 21** | 500±23 (4/4) | >650 (0/6) | 598±36 (7/7) |
| **Sheep-Sc Langlade** | >650 (3/3) | >650 (0/6) | >630 (5/5) |
| **Goat-BSE1** | 497±31 (5/5) | 519±42(5/5) | 533±44 (5/5) |
| **Goat-BSE2** | 484±34 (7/7) | 478±31(4/4) | 470±14 (5/5) |
| **Cattle-BSE** | 583±57 (6/6) | >650 (0/6) | 617±47 (5/5) |

[a] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

[b] Heterozygous Q/$K_{222}$ mice were obtained by crossbreeding Wt-Tg501 and $K_{222}$-Tg516 mice.

When inoculated with Cattle-BSE, heterozygous Q/$K_{222}$ animals had longer ST than Wt-Tg501 mice (Table 13). Nevertheless, goat-BSE isolates exhibited similar transmission features in all challenged Tg mouse models (W-Tg501, $K_{222}$-Tg516, and Q/$K_{222}$ mice) regardless of the expressed $PrP^C$ variant.

Histopathological studies confirm the presence of $PrP^{res}$ and spongiform changes in the brains of heterozygous Q/$K_{222}$ mice infected with Goat-Sc F2, Goat-Sc F10, Goat-Sc S3, Sheep-Sc 21, Sheep-ScLanglade, and goat and cattle-BSE isolates. $PrP^{res}$ deposits and vacuolation profiles in these mice were similar but not identical to those from Wt-Tg501 mice infected with the same isolates (data not shown). However, we cannot rule out the possibility that these slight differences are in some way connected to the old age of Q/$K_{222}$ mice that had longer survival times than Wt-Tg501.

In order to determine which allele was responsible for the replication of the classical scrapie and cattle-BSE isolates in heterozygous Q/$K_{222}$ mice, brain homogenate from Goat-Sc F2-infected Q/$K_{222}$ mice was IC inoculated in both hemizygous goat Wt-Tg501 and hemizygous $K_{222}$-Tg516 mice.

**Table 19.** Transmission of Goat-Sc F2-passaged in Q/$K_{222}$ heterozygous mice to Wt-Tg501 and $K_{222}$-Tg516 mice

| | **Mean survival time in days ± SEM ($n/n_0$)[a]** | |
|---|---|---|
| **Isolate** | **Wt-Tg501 ($Q_{222}$/-) 1X[b]** | **$K_{222}$-Tg516 ($K_{222}$/-) 1X** |
| **Goat-Sc F2/Tg511[c]** | 225±12 (5/5) | >650 (0/5) |
| **Goat-Sc F2** | 250±36 (4/4) | >650 (0/7) |

[a] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

[b] $PrP^C$ level of expression in the brain compared to $PrP^C$ in goat and sheep brains as assessed by Western blot analysis (Fig. 13A and E).

[c] Pool of terminally ill Q/$K_{222}$ heterozygous mice inoculated with Goat-Sc F2 isolate.

None of the hemizygous $K_{222}$-Tg516 mice succumbed to the inoculation with Goat-Sc F2/Tg511 isolate (Table 19). $K_{222}$-Tg516 mice were sacrificed at the end of their lifespan and had neither clinical signs nor $PrP^{res}$ in their brains when assessed by WB analysis (data not shown). By contrast, all the hemizygous goat

Wt-Tg501 mice were infected with this isolate and had similar ST to the Wt-Tg501 inoculated with the original Goat-Sc F2 (Table 19).

## 2. Assessment of the resistance/susceptibility of polymorphic variants of the prion protein to prion infection in natural host (goats)

In an attempt to validate our Tg mice as a model for studying the role of goat $PrP^C$ polymorphic variants in resistance/susceptibility to prion infection in the natural host (goats), we worked in collaboration with two different research groups in the framework of the StrepBSE in goat EU project (UE-FP6-2005-FOOD4B-036353). Dr. Olivier Andréoletti headed the experimental scrapie transmissions to goats at the École Nationale Vétérinaire (ENV-INRA) in Toulouse (France), while Dr. Martin Groshup´s group performed the oral Goat-BSE inoculations in goats at the Institute for Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut (FLI), Insel Riems (Germany).

### 2.1. Susceptibility of WT, I/$M_{142}$, R/$H_{154}$, R/$Q_{211}$, and Q/$K_{222}$ goats to oral and intracerebral infection with classical scrapie

Goats harboring different *prnp* genotypes (WT: $I_{142}R_{154}R_{211}Q_{222}$/$I_{142}R_{154}R_{211}Q_{222}$ or *prnp* variants $M_{142}$, $H_{154}$, $Q_{211}$ or $K_{222}$) in either homozygosis or heterozygosis were challenged with a natural scrapie isolate (see Materials and Methods, section 2.2.1 for more information about the inoculum) via intracerebral (IC) or oral routes in the ENV-INRA in Toulouse (France) following the procedures described in Materials and Methods, section 2.2.1. For oral challenges, goat kids received the inoculum (scrapie-infected brain) by natural suckling within 48 hours of birth and a second dose at the age of 30 days. For IC challenges, inoculum (the same homogenized scrapie-infected brain) was injected into the temporal cortex of six-month-old goats. Both groups of animals were monitored for the development of neurological diseases and euthanized at the clinical stage of the disease. The presence of $PrP^{Sc}$ was assessed in brains by WB and IHC analysis. The effect of each polymorphism on the susceptibility of goats to

scrapie was assessed and compared to the outcomes obtained in Tg mice. This bioassay also permitted us to analyze the effect of the inoculation route in prion transmission.

Results indicated that all the WT goats were susceptible to the classical scrapie infection, both via oral and IC inoculation routes (Table 20). Goats developed neurological signs of scrapie disease – mainly motor alterations – and $PrP^{Sc}$ was detected in their brains by WB and IHC analysis (data not shown).

**Table 20.** Scrapie incubation periods in goats inoculated via oral and intracerebral routes according to their genotypes at codon 142, 154, 211 and 222 of the *prnp*

| | **Incubation period in days ± SEM ($n/n_0$)[b]** | |
|---|---|---|
| ***prnp genotype*** | **Oral inoculation** | **Intracerebral inoculation** |
| wt [a] | 1141±93 (9/9) | 486±21 (5/5) |
| wt; I/ $M_{142}$ | 1490±126 (4/4) | 788±99 (5/5) |
| wt; R/ $H_{154}$ | >2500 (0/6) | 624±148 (5/5) |
| wt; R /$Q_{211}$ | >2500 (0/6) | 1291±325 (5/5) |
| wt; Q /$Q_{211}$ | NA | 770±139 (10/10) |
| wt; Q /$K_{222}$ | >2500 (0/5) | 1900, 2174 (2/5) |
| wt; K /$K_{222}$ | NA | 2101 (1/5) |

[a] Goats were sequenced following the procedures described in Materials and Methods (see section 2.1.1). Wild type (wt) $PrP^C$ sequence ($I_{142}R_{154}R_{211}Q_{222}$/$I_{142}R_{154}R_{211}Q_{222}$) and the polymorphic variants observed are indicated.

[b] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

NA: Not analyzed.

As in the case of the Wt goats, all goats harboring the I/$M_{142}$ *prnp* genotype developed a clinical TSE, although with some delays in the disease onset in comparison to WT goats (Table 20). These prolonged incubation periods were reported in both oral and IC challenges and agree with the delays in ST that we previously described for scrapie-infected $M_{142}$-Tg541 mice (Table 15), which further reinforces the idea that $M_{142}$-$PrP^C$ polymorphic variant is not associated with resistance to classical scrapie infection.

On the other hand, none of the goats harboring the $H_{154}$, $Q_{211}$ or $K_{222}$-$PrP^C$ variants succumbed to the oral scrapie inoculation after over 2500 days. None of these goats showed any sign of prion disease and were culled due to

intercurrent diseases (Table 20). $PrP^{Sc}$ was not detected by WB or IHC analysis of the brains of any of these goats. Unlike the oral challenges, the heterozygous $R/H_{154}$ and $R/Q_{211}$ goats – as well as the $Q/Q_{211}$ homozygous animals that were IC inoculated – developed a clinical TSE with much longer incubation periods than IC-inoculated WT goats (Table 20). Interestingly, only two heterozygous goats and one homozygous $K_{222}$ goat developed prion disease after an IC challenge, all with four-to-five times longer incubation times than WT goats. These delays in incubation periods compared to IC inoculated WT goats agree with the prolonged ST and resistance registered for $Q_{211}$-Tg580 and $K_{222}$-Tg516 mice IC-inoculated with different classical scrapie strains (Tables 12 and 13).

### 2.2. Susceptibility of WT, $R/Q_{211}$, and $Q/K_{222}$ goats to oral infection with goat-BSE

Goats harboring WT ($R_{211}Q_{222}/R_{211}Q_{222}$), $R/Q_{211}$, or $Q/K_{222}$ *prnp* genotypes were orally inoculated with a BSE adapted to goat isolate (Foster et al., 1993; Goldmann et al., 1996) (see Table 6 in Materials and Methods for more information about the inoculum) and were monitored for the development of prion disease. Goats were euthanized at the clinical stage or at scheduled times during the incubation period to follow:

- Disease onset, neurological lesions and $PrP^{Sc}$ deposition at FLI, InselRiems (see Materials and Methods, sections 2.2.1-2.2.3).
- Presence of $PrP^{res}$ and prion infectivity in brain and/or certain peripheral tissues at CISA-INIA, Madrid (see Materials and Methods, sections 2.2.4 and 2.3).

By combining all these results, the relative susceptibility of each goat *prnp* genotype to oral infection with Goat-BSE was assessed and compared to that obtained from goat-PrP Tg mice. In this way, we were able to determine the reliability of the results obtained in BSE transmission studies in goat PrP-Tg mice.

### 2.2.1 Onset of clinical signs and $PrP^{Sc}$ accumulation

None of the WT goats killed at either six or 12 mpi exhibited any signs associated with BSE disease (Table 26) in the brain and the peripheral tissues examined here, neither by histopathology and Immunohistochemistry (IHC) nor by Western-blot (WB) analysis. Clinical signs of the disease were first observed in a WT goat (ZG01) at 24 mpi (Table 21). This animal revealed clear neurological signs and a spongiform encephalopathy associated with distinct accumulation of $PrP^{Sc}$ in the brain stem as demonstrated by Hematoxylin-Eosin (HE) and immunohistochemistry analysis. WB (Fig. 24) analysis confirmed the presence of BSE-derived $PrP^{res}$ in this goat's brain. However, in the lymph node (LN) poplitealis, retractor bulbi muscle or psoas major muscle all methods applied failed to detect any amount of $PrP^{Sc}$ (Table 21).

In contrast to WT goats, WB, IHC, and HE analysis of one $R/Q_{211}$ goat killed at 24 mpi revealed the absence of any $PrP^{Sc}$ deposits and neurodegenerative alterations in its brain (Table 21). However, three $R/Q_{211}$ goats reached the clinical phase of BSE disease at 33 (ZG28), 34 (ZG05), and 36 (ZG20) mpi (Table 21) associated with clear spongiform encephalopathy and accumulation of $PrP^{Sc}$ as shown in IHC analysis. Additionally, all three $R/Q_{211}$ goats (33–36 mpi) were scored as $PrP^{res}$ positive in their brains by WB analysis (Fig. 24) but not in their muscles or popliteal LN, again similar to the WT goats. Together, these results indicate that the $Q_{211}$-$PrP^{C}$ variant has a non-substantial effect on the susceptibility of goats to oral infection with Goat-BSE since this effect only extends the incubation periods of the disease.

**Table 21.** Clinical sign evaluation and $PrP^{Sc}$ detection in tissues of WT, $R/Q_{211}$, and $Q/K_{222}$ goats orally challenged with a Goat-BSE isolate and then sequentially culled

| *prnp* genotype | Goat code | Endpoint (mpi) | Clinical signs | Goat tissue | WB $PrP^{res}$ [d] | IHC $PrP^{Sc}$ [e] |
|---|---|---|---|---|---|---|
| WT[a] | ZG26 | 6 | - | brain | - | - |
| | | | | m[b]. psoas major | - | NA |
| | | | | m.retractorbulbi | - | NA |
| | | | | popliteal LN[c] | - | NA |
| | ZG32 | 6 | - | brain | - | - |
| | | | | m. psoas major | - | NA |
| | | | | m.retractorbulbi | - | NA |
| | | | | popliteal LN | - | NA |
| | ZG35 | 6 | - | brain | - | - |
| | | | | m. psoas major | - | NA |
| | | | | m.retractorbulbi | - | NA |
| | | | | popliteal LN | - | NA |
| | ZG19 | 12 | - | brain | - | - |
| | | | | m. psoas major | - | NA |
| | | | | m.retractorbulbi | - | NA |
| | | | | popliteal LN | - | NA |
| | ZG24 | 12 | - | brain | - | - |
| | | | | m. psoas major | - | NA |
| | | | | m.retractorbulbi | - | NA |
| | | | | popliteal LN | - | NA |
| | ZG30 | 12 | - | brain | - | - |
| | | | | m. psoas major | - | NA |
| | | | | m.retractorbulbi | - | NA |
| | | | | popliteal LN | - | NA |
| | ZG01 | 24 | + | brain | + | + |
| | | | | m. psoas major | - | - |
| | | | | m.retractorbulbi | - | - |
| | | | | popliteal LN | - | - |
| $R/Q_{211}$ | ZG13 | 24 | - | brain | - | - |
| | ZG28 | 33 | + | brain | + | + |
| | | | | m. psoas major | - | - |
| | | | | m.retractorbulbi | - | - |
| | | | | popliteal LN | - | - |
| | ZG05 | 34 | + | brain | + | + |
| | | | | m. psoas major | - | - |
| | | | | m.retractorbulbi | - | - |
| | | | | popliteal LN | - | - |
| | ZG20 | 36 | + | brain | + | + |
| | | | | m. psoas major | - | - |
| | | | | m.retractorbulbi | - | - |
| | | | | popliteal LN | - | - |
| $Q/K_{222}$ | ZG10 | 24 | - | brain | - | - |
| | ZG25 | 44 | - | brain | - | - |
| | | | | m. psoas major | - | - |
| | | | | popliteal LN | - | - |
| | ZG11 | 45 | - | brain | - | - |
| | | | | m. psoas major | - | - |
| | | | | popliteal LN | - | - |
| | ZG03 | 86 | - | brain | - | - |
| | | | | m. psoas major | - | - |
| | | | | m.retractorbulbi | - | - |
| | | | | popliteal LN | - | - |

See next page for footnotes.

[a] Goats were sequenced following the procedures described in Materials and Methods (see section 2.1.1). Wild type (wt) $PrP^C$ sequence ($R_{211}Q_{222}/R_{211}Q_{222}$); the polymorphic variants observed are indicated.

[b] Muscle.

[c] Limphonode.

[d] Brain $PrP^{res}$ was detected by Western blot using Sha31 mAb (Fig. 24).

[e] Brain $PrP^{Sc}$ was detected by Immunohistochemistry (IHC) as described in Materials and Methods section 2.2.3.

NA: Not analyzed

None of the $Q/K_{222}$ goats killed at 24, 44 and 45 mpi showed any evidence of prion disease (Table 21). Moreover, all of these goats were scored as $PrP^{Sc}$ negative for all of their analized tissues by IHC and WB (Table 21) thereby suggesting that $K_{222}$-$PrP^C$ variant provides goats with resistance to the oral infection with goat-BSE. These results contrast with the total susceptibility of the Tg mice expressing this polymorphic variant ($K_{222}$-Tg516 mice) to IC infection with Goat-BSE prions (Table 12). $K_{222}$-Tg516 mice had very similar ST to goat Wt-Tg501 mice, which suggests that the $K_{222}$-$PrP^C$ variant does not affect susceptibility to Goat-BSE. Thus, non-genetic factors such as the inoculation route are the probable origin of the resistance of $Q/K_{222}$ goats to oral infection with Goat-BSE.

WB analysis of the brain $PrP^{Sc}$ in both WT- and $R/Q_{211}$-infected goats showed a typical BSE $PrP^{Sc}$ banding pattern characterized by a 19K unglycosilated band and prominent diglycosylated species. The glycoprofiles were indistinguishable from those produced by the goat-BSE isolate originally used in the goat challenges (Fig. 24).

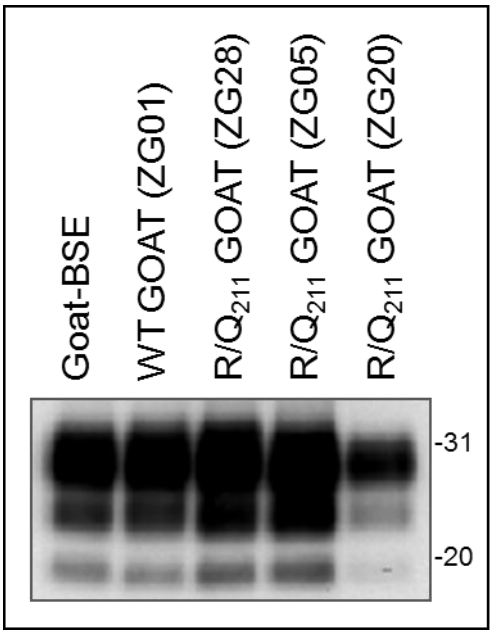


**Figure 24. Brain $PrP^{res}$ from wild type (WT) and $R/Q_{211}$ goats orally inoculated with a goat-BSE isolate.** Immunoblots of brain $PrP^{res}$ detected with Sha31 mAb. Identical amounts of 10% brain homogenate were loaded in each lane. The original Goat-BSE isolate used for goat inoculations (Foster et al., 1993; Goldmann et al., 1996) was also included in the blot (Goat-BSE). Molecular weights in kilodaltons are shown on the right-hand side of the blot.

### 2.1.2. Infectivity in different tissues from inoculated goats

#### *2.1.2.1. Titration of the goat-BSE isolate used for goat inoculations*

$PrP^{Sc}$ is a specific and widely used TSE marker. Nevertheless, its sensitivity can be lower than the infectivity measured by mouse bioassay. In order to select the most sensitive mouse bioassay, the goat-BSE isolate originally used for the goat-transmission inoculations (Foster et al., 1993; Goldmann et al., 1996) was titrated in both BoPrP-Tg110 and WT-Tg501 mice by end-point dilution. BoPrP-Tg110 and WT-Tg501 mice were intracerebrally inoculated with 20µl of 10-fold serial dilutions of the goat-BSE isolate (10% brain homogenate); the infectious titer of the isolate was calculated by the Reed-Muench method (see Materials and Methods, section 2.3.2).

**Table 22.** Titration of the Goat-BSE isolate by end-point titration in both BoPrP-Tg110 and goat Wt-Tg501 mice

| Inoculum dilution | Equivalent grams of inoculated brain[a] | Mean survival time in days ± SEM ($n/n_0$)[b] | |
|---|---|---|---|
| | | BoPrP-Tg110[c] | Wt-Tg501 (1X)[d] |
| | 0,002 (2 x $10^{-3}$) | 239±66 (6/6) | 381±43 (6/6) |
| $10^{-1}$ | 0,0002 (2 x $10^{-4}$) | 289±24 (6/6) | 367±12 (6/6) |
| $10^{-2}$ | 0,00002 (2 x $10^{-5}$) | 345±57 (6/6) | 408±24 (6/6) |
| $10^{-3}$ | 0,000002 (2 x $10^{-6}$) | 373±29 (6/6) | 528±64 (3/7) |
| $10^{-4}$ | 0,0000002 (2 x $10^{-7}$) | 351±14 (4/6) | >600 (0/6) |
| $10^{-5}$ | 0,00000002 (2 x $10^{-8}$) | 441, 450 (2/6) | >600 (0/6) |
| **Infectious titer of the Goat-BSE isolate[e]** | | **1.58 x $10^7$ ID/g** | **3.79 x $10^5$ ID/g** |

[a] Grams of Goat-BSE-infected brain inoculated in each IC dose (20µl of brain homogenate per mouse).

[b] $n/n_0$: diseased, $PrP^{res}$: positive/inoculated animals.

[c] Transgenic mice expressing the bovine $PrP^C$ sequence (Castilla et al., 2003).

[d] $PrP^C$ level of expression in the brain compared to $PrP^C$ in goat and sheep brain as assessed by Western blot analysis (Fig. 13A).

[e] Infectious titer calculated by the Reed-Muench method and expressed as ID per grams (ID/g) of Goat-BSE-infected brain inoculated in each mouse model.

BoPrP-Tg110 mice were selected for detecting prion infectivity in goat tissues because of their greater sensitiveness to the prion infectivity detection of the Goat-BSE isolate than Wt-Tg501 mice (Table 22). Then, a regression curve was drawn with the dilutions of the Goat-BSE isolate that produced 100% attack rates in BoPrP-Tg110 mice (Fig. 25). Finally, an equation fitting the data was obtained ($y = 288{,}12e^{-0{,}049x}$) and used to obtain the infectious titers (y) in the goat tissues from their survival times values (x) (Table 22).

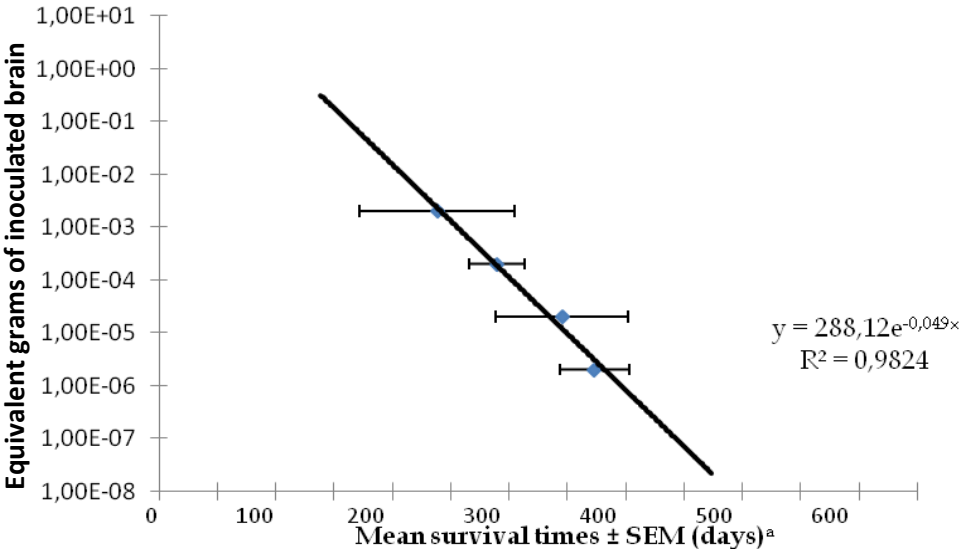


**Figure 25. Titration of the Goat-BSE isolate (Foster et al., 1993; Goldmann et al., 1996) by end-point dilution in BoPrP-Tg110 mice**. [a]Mean survival times in days± standard error of the mean (n/n0: diseased, $PrP^{res}$positive/inoculated animals) are represented on the x-axis of the plot and the equivalent grams of the inoculated Goat-BSE-infected brain on the y-axis.

### *2.1.2.2 Relative infectivity in tissues from goats inoculated with goat-BSE*

No prion infectivity was detected in any tissue from WT goats killed at 6 or 12 mpi. When their brain and peripheral tissue homogenates were inoculated into BoPrP-Tg110 mice, none of the mice succumbed to prion disease. All were euthanized at 650 dpi and none had any clinical signs or $PrP^{res}$ in their brains by WB analysis. Prion infectivity was detected in the brain of a WT goat killed at 24 mpi (ZG01 goat). After inoculation, BoPrP-Tg110 mice had 100% AR and short ST (261±20 dpi) (Table 23), which correspond to a highly infectious titer ($6.35 \times 10^6$ ID/g). Interestingly, BoPrP-Tg110 mice also succumbed to the inoculation of $PrP^{Sc}$ negative popliteal LN, psoas major muscle, and retractor bulbi muscle homogenates from this goat, and had longer ST and lower AR than those obtained with the brain homogenate (Table 23).

**Table 23.** Infectious titer in tissues from Goat-BSE orally inoculated WT, $R/Q_{211}$, and $Q/K_{222}$ *prnp* goats as determined by mouse bioassay

| *prnp* genotype | Goat codes of pooled tissues | End point (mpi) | Goat tissue | Mouse bioassay in BoPrP-Tg110 mice | |
|---|---|---|---|---|---|
| | | | | **ST±SEM ($n/n_0$)[b]** | **Infectious titer[c]** |
| WT[a] | ZG26-ZG32-ZG35 | 6 | brain | 650 (0/6) | <5x $10^2$ [d] |
| | | | psoas major muscle | 650 (0/6) | <5x $10^2$ |
| | | | retractor bulbi muscle | 650 (0/6) | <5x $10^2$ |
| | | | popliteal lymph node | 650 (0/6) | <5x $10^2$ |
| | ZG19-ZG24-ZG30 | 12 | brain | 650 (0/6) | <5x $10^2$ |
| | | | psoas major muscle | 650 (0/6) | <5x $10^2$ |
| | | | popliteal lymph node | 650 (0/6) | <5x $10^2$ |
| | ZG01 | 24 | brain | 261±20 (6/6) | 6.35 x $10^6$ |
| | | | psoas major muscle | 596, 598 (2/6) | <5x $10^2$ |
| | | | retractor bulbi muscle | 469±29 (3/6) | <5x $10^2$ |
| | | | popliteal lymph node | 526 (1/6) | <5x $10^2$ |
| $R/Q_{211}$ | ZG13 | 24 | brain | 650 (0/6) | <5x $10^2$ |
| | ZG28-ZG05-ZG20 | 33-34-36 | brain | 258±15 (6/6) | 7.36 x $10^6$ |
| | | | psoas major muscle | 334±24 (6/6) | 1.78 x $10^5$ |
| | | | retractor bulbi muscle | 307±84 (5/5) | 6.67 x $10^5$ |
| | | | popliteal lymph node | 406±50 (2/4) | <5x $10^2$ |
| $Q/K_{222}$ | ZG10 | 24 | brain | 650 (0/6) | <5x $10^2$ |
| | ZG25 | 44 | brain | 650 (0/6) | <5x $10^2$ |
| | | | psoas major muscle | 650 (0/6) | <5x $10^2$ |
| | | | popliteal lymph node | 650 (0/6) | <5x $10^2$ |
| | ZG11 | 45 | brain | 400±50 (6/6) | 7.00 x $10^3$ |
| | | | psoas major muscle | 522 (1/6) | <5x $10^2$ |
| | | | popliteal lymph node | 650 (0/6) | <5x $10^2$ |
| | ZG03 | 86 | brain | ongoing | ND |
| | | | psoas major muscle | ongoing | ND |
| | | | retractor bulbi muscle | ongoing | ND |
| | | | popliteal lymph node | ongoing | ND |

[a] Goats were sequenced following the procedures described in Materials and Methods (see section 2.1.1). Wt type (wt) $PrP^{Sc}$ sequence: $A_{136}R_{154}Q_{171}/A_{136}R_{154}Q_{171}$.

[b] Mean Survival Time in days ± Standard error of the mean (n/n0: diseased, $PrP^{res}$positive/inoculated animals).

[c] The infectious titer of each goat tissue was calculated as a function of the survival times obtained after their inoculation in BoPrP-Tg110 mice and expressed as ID per grams of the inoculated tissue (ID/g) (see supplemental data).

[d] Limit of detection of the mouse bioassay calculated as ID/g of inoculated brain in each 20ul of inoculated homogenate using the Reed-Muench method.

Prion infectivity was not detected in the brain of the R/$Q_{211}$ goat killed at 24 mpi (Table 23). However, all BoPrP-Tg110 mice succumbed to the inoculation of the pooled brain homogenates from ZG28, ZG05, and ZG20 R/$Q_{211}$ goats killed at 33–36 mpi. These mice had similar ST (258±15 dpi) to those previously obtained from the inoculation of the ZG01 WT goat brain and therefore their infectious titers were comparable (7.36 x $10^6$ ID/g and 6.35 x $10^6$ ID/g, respectively). Like the WT goat, R/$Q_{211}$ goats also had prion infectivity in their $PrP^{Sc}$ negative peripheral tissues. Pooled homogenates from popliteal LN, psoas major muscle, and retractor bulbi muscle infected BoPrP-Tg110 mice with higher AR and lower ST than peripheral tissues from ZG01 WT goat (Table 23).

Only the brain from a Q/$K_{222}$ goat killed at 45 mpi (ZG11) had a low infectivity (Table 29), while the brain from a Q/$K_{222}$ goat killed at 44 mpi (ZG25) harbored no prion infectivity. All the BoPrP-Tg110 mice succumbed to the brain inoculation from goat ZG11, albeit with considerably longer ST (400±50 dpi) than those obtained with brains from WT (261±20 dpi) and R/$Q_{211}$ (258±15 dpi) goats. Traces of infectivity were also detected in $PrP^{Sc}$ negative muscle psoas major from both Q/$K_{222}$ goats with a low AR (1/6) and very long ST (550 and 522 dpi) in BoPrP-Tg110 mice. Tissues from the Q/$K_{222}$ goat culled at 76 mpi have recently been inoculated in BoPrP-Tg110 mice but with no results as yet (Table 23).

In all cases, brain $PrP^{res}$ patterns from infected BoPrP-Tg110 mice were similar to those observed in the original goat-BSE isolate (Foster et al., 1993; Goldmann et al., 1996), as well as to those found in goat tissue homogenates, regardless of the goat *prnp* genotype (WT, R/$Q_{211}$, or Q/$K_{222}$) or the inoculated tissue (Fig. 26).

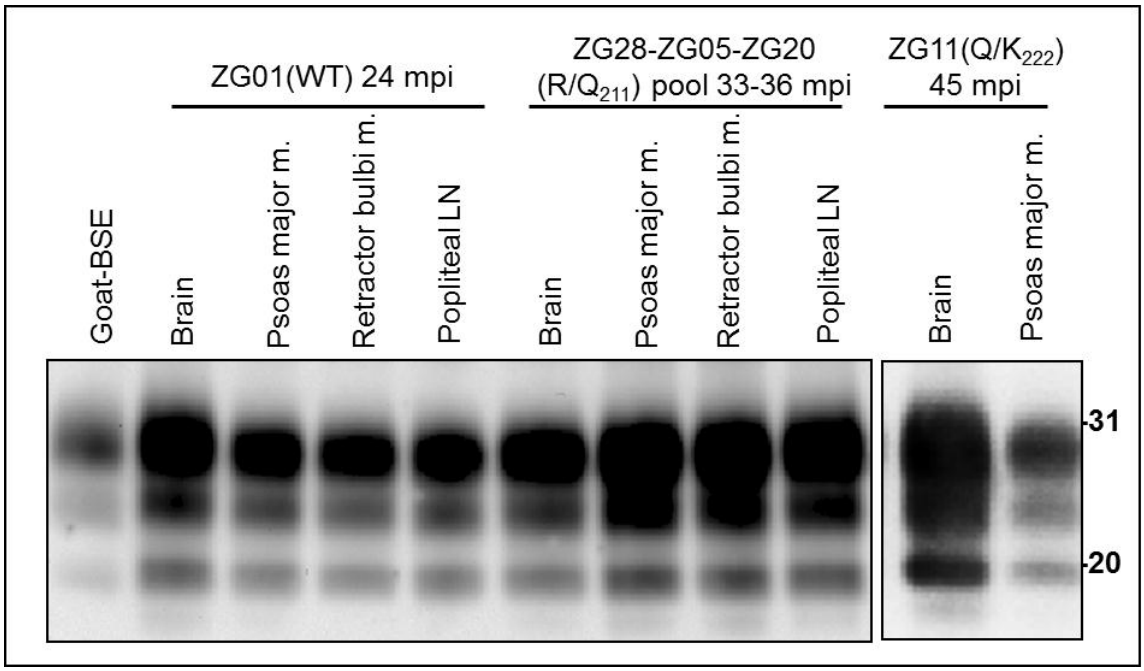


**Figure 26. Brain $PrP^{res}$ detected in BoPrP-Tg110 mice inoculated with different goat tissue homogenates.** Immunoblots of $PrP^{res}$ in BoPrP-Tg110 mice inoculated with different tissue homogenates from ZG01 WT ($R_{211}Q_{222}$/RQ) goat, ZG28, ZG05 and ZG20 R/$Q_{211}$ goats (pooled homogenates), and ZG11 Q/$K_{222}$ goat. Similar quantities of $PrP^{res}$ were loaded to enable adequate comparison; immunoblots were detected with Sha31 mAb. The original goat-BSE isolate used for goat inoculations (Foster et al., 1993; Goldmann et al., 1996) was also included in the blot (Goat-BSE). Molecular weights in kilodaltons are shown on the right-hand side of the blot. M: muscle; LN: lymph node.

# VII. DISCUSSION

A fundamental event in the pathogenesis of prion diseases is the misfolding of host cellular prion protein ($PrP^{C}$) into pathological prion protein ($PrP^{Sc}$). It is well known that certain amino acid changes in the $PrP^{C}$ sequence can affect the efficiency of $PrP^{Sc}$ replication and prion transmission. Besides the PrP amino acid differences between host $PrP^{C}$ and donor $PrP^{Sc}$, prion strains also modulate prion conversion, possibly as a result of the variety of conformations that $PrP^{Sc}$ can adopt. It is important therefore to study PrP sequence changes in combination with prion strains as a way of understanding the molecular mechanisms governing the susceptibility/resistance to prion infection.

In this work we generated a panel of transgenic (Tg) mouse lines expressing either the goat wild type $PrP^{C}$ or this $PrP^{C}$ with single amino acid exchanges corresponding to goat or sheep $PrP^{C}$ polymorphic variants ($I/M_{142}$, $R/H_{154}$, $R/Q_{211}$, $Q/K_{222}$, $Q/R_{171}$, and $N/K_{176}$) that were subsequently challenged with several prion strains. Their susceptibilities were assessed and compared to determine the individual effect of each $PrP^{C}$ variant in the resistance/susceptibility to prion infection. At the same time as these transmission studies in Tg mice were being carried out, experimental goat inoculations with either a scrapie field case or a goat-BSE isolate were performed thanks to a collaborative work in ENVT (Toulouse) and FLI (Insel Riems), respectively, and their results were compared with those obtained in the transmission studies in Tg mice. In this way, we were able to confirm in the natural host (goats) the reliability of our results for mouse models and to evaluate the effect of $PrP^{C}$ polymorphic variants on the susceptibility of goats to prion infection.

Tg mouse lines expressing different polymorphic variants of the $PrP^{C}$ ($M_{142}$, $H_{154}$, $R_{171}$, $K_{176}$, $Q_{211}$, and $K_{222}$) were generated by directed mutagenesis of the pMo-GoPrP.Xho plasmid used for generating the goat Wt-Tg501 mouse line. Of these mouse lines, we selected at least one founder expressing a physiological brain $PrP^{C}$ level similar and/or comparable to that in goats. The only exception was $H_{154}$-Tg563, for which no founder expressing more than 0.25-fold the $PrP^{C}$

(0.25X) in the goat brain was obtained, despite the large number of microinjections performed (Table 8); this suggests that higher levels of $H_{154}$-$PrP^C$ could be toxic. The hemizygous $Q_{211}$-Tg580 (0.5X) mouse line was bred to homozygosis in order to obtain mice with a physiological brain $PrP^C$ level comparable to hemizygous Wt-Tg501 mice (1X). Finally, the hemizygous $K_{176}$-Tg570 (2X) mouse line had a similar brain $PrP^C$ level to that of the homozygous Wt-Tg501 mice (2X). Selected mouse lines were healthy and exhibited physiological behavior until the end of their lifespans; no histopathological lesion due to prion disease was observed in their brains, thereby ruling out the possibility of spontaneous disease development as a result of the expression of the mutated $PrP^C$. Together, this collection of mouse lines is deemed to be suitable for studying the role of polymorphic variants in goat and sheep $PrP^C$ in the susceptibility to prion infection.

**1. Susceptibility of goat WT-PrP Tg501 mice to scrapie and BSE infection**

Wt-Tg501 mice were susceptible to all tested scrapie and BSE isolates regardless of the strain (classical or atypical scrapie, and classical or atypical BSE), geographical origin, $PrP^{Sc}$ genotype, $PrP^{res}$ Western blot (WB) profile, or behavior upon passage in BoPrP-Tg110 mice of the prion isolates (Table 11). However, transmission features clearly varied in terms of these factors (see below).

Brain $PrP^{res}$ glycoprofile of the classical scrapie-infected Wt-Tg501 mice analyzed using WB demonstrated that our Wt-Tg501 mice largely reproduce the WB signature of the inoculated isolate (Fig. 16A). Most of these isolates appeared to give a molecular glycoprofile with a 21K unglycosilated band that was fairly well maintained in the Wt-Tg501 mouse brain, except for Goat-Sc UKA2 where some infected mouse brains showed a 21K unglycosilated band, while others exhibited a double 19-21K unglycosilated band. These findings point to the coexistence of two different agents in this isolate. Finally, two isolates (Goat-Sc F14 and Goat-Sc UKB2) maintained their double 19-21K unglycosilated band after passage in the Wt-Tg501 mouse brain.

Other transmission features in Wt-Tg501 mice revealed additional differences in the panel of classical scrapie isolates. Highly variable ST (Table 11) and neuropathological alterations (Fig. 17) were obtained, which indicates the existence of at least three types of classical scrapie isolates: "Italian" isolates, "non-Italian fast" isolates, and "non-Italian slow" isolates. "Italian" isolates displayed the longest ST (550–650 dpi) (Table 11) and shared particular histopathological alterations (Fig. 17) that differed from the other isolates (non-Italian). Among the "non-Italian" isolates, "fast" (<300 dpi after second passage) and "slow" (>300 dpi after second passage) isolates could be identified irrespective of their geographical origin (Table 11). Histolopathological analysis (Fig. 17) revealed a specific and distinct neuropathological pattern for isolates belonging to each of these two groups (fast or slow). Within "non-Italian fast" isolates two different subgroups could be differentiated on the basis of the molecular weight of the brain $PrP^{res}$ unglycosilated band (21K or 19-21K) (Table 11). Taken together, the homogeneity of the transmission features within each group suggests that the different isolates included in each group belong to the same scrapie strain and so all our panel might include at least four classical scrapie strains.

Transmission experiments in BoPrP-Tg110 mice –and notably, the analysis of the brain $PrP^{res}$ glycoprofile in BoPrP-Tg110 mice– revealed additional information that helps type classical scrapie isolates (Table 24). An analysis of this feature clearly distinguishes two groups of scrapie isolates: those that showed a 19K unglycosilated band and those that produced a 21K band (Fig. 15A). Interestingly, all Italian isolates transmitted to BoPrP-Tg110 mice had a brain $PrP^{res}$ 21K unglycosilated band in this mouse model (Table 24), which provides further supporting evidence for the existence of an "Italian" classical scrapie strain that differs from the rest of scrapie isolates inoculated. The absence of transmission of two "Italian" isolates (Goat-Sc I4 and Goat-Sc I12) to BoPrP-Tg110 mice could be the result of a combination of a low infectious titter of these isolates and the high transmission barrier of these mice to all Italian isolates.

**Table 24.** Summary of the survival times, attack rates and brain $PrP^{res}$ WB profile of classical scrapie isolates obtained during the transmission studies in goat Wt-Tg501 and BoPrP-Tg110 mouse lines

| Isolate | | Transmission in Wt-Tg501 mice | | | | Transmission inBoPrP-Tg110 mice | | | | Group |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1st passage | | 2nd passage | | 1st passage | | 2nd passage | | |
| Name | $PrP^{res}$ profile[a] | ST ± SEM ($n/n_0$)[b] | $PrP^{res}$ profile | ST ± SEM ($n/n_0$)[c] | $PrP^{res}$ profile | ST ± SEM ($n/n_0$) | $PrP^{res}$ profile | ST ± SEM ($n/n_0$) | $PrP^{res}$ profile | |
| **Goat-Sc I2** | 21K | 526±13 (6/6) | 21K | **>600 (ongoing)** | - | 453 (1/6) | 21K | **167±9 (7/7)** | **21K** | I ($Tg501VS_{21K}$ / $Tg110F_{21K}$) |
| **Goat-Sc I3** | 21K | >650 (5/5) | 21K | **>600 (ongoing)** | - | 464 (1/7) | 21K | **163±16 (7/7)** | **21K** | |
| **Goat-Sc I4** | 21K | 582±20 (6/6) | 21K | **>350 (ongoing)** | **21K** | >650 (0/5) | - | **NA** | **NA** | |
| **Goat-Sc I9** | 21K | 578±25 (5/5) | 21K | **547±25 (6/6)** | **21K** | 324±90 (6/6) | 21K | **174±8 (5/5)** | **21K** | |
| **Goat-Sc I12** | 21K | 591±42 (4/4) | 21K | **>350 (ongoing)** | **21K** | >650 (0/7) | - | **NA** | - | |
| **Goat-Sc F16** | 21K | 551±89 (3/3) | 21K | **242±26 (5/5)** | **21K** | 348±58 (3/6) | 21K | **177±9 (6/6)** | **21K** | I ($Tg501F_{21K}$ / $Tg110F_{21K}$) |
| **Goat-Sc F2** | 21K | 259±28 (4/4) | 21K | **212±16(6/6)** | **21K** | 343±163 (4/5) | 19K | **198±13 (6/6)** | **19K** | NI ($Tg501F_{21K}$ / $Tg110F_{19K}$) |
| **Goat-Sc F3** | 21K | 287±14 (6/6) | 21K | **228±7 (6/6)** | **21K** | 290±48 (3/6) | 19K | **191±3 (6/6)** | **19K** | |
| **Goat-Sc F6** | 21K | 468±15 (4/4) | 21K | **299±6 (6/6)** | **21K** | 523±166 (6/6) | 19K | **178±13 (5/5)** | **19K** | |
| **Goat-Sc N3** | 21K | 451±9 (4/4) | 21K | **254±50 (6/6)** | **21K** | 324, 703 (2/6)* | 19K | **203±7 (5/5)** | **19K** | |
| | | | | | | | | **493±72 (6/6)** | **19K** | |
| **Goat-Sc S2** | 21K | 228±15 (6/6) | 21K | **233±4 (6/6)** | **21K** | 384±149 (6/6) | 19K | **237±39 (9/9)** | **19K** | |
| **Goat-Sc S3** | 21K | 221±16 (6/6) | 21K | **233±64 (5/5)** | **21K** | 271±19 (6/6) | 19K | **254±52 (4/4)** | **19K** | |
| **Sheep-Sc 21** | 21K | 194±5 (6/6) | 21K | **205±18 (6/6)** | **21K** | 244±13 (6/6) | 19K | **187±2 (4/4)** | **19K** | |
| **Sheep-Sc Langlade** | 21K | >650 (3/3) | 21K | **222±5 (6/6)** | **21K** | 504±47 (6/6) | 19K | **229±37 (4/4)** | **19K** | |
| **Sheep-Sc 09** | 21K | 277±31 (5/5) | 21K | **NA** | **NA** | 230±66 (4/6) | 19K | **168±6 (6/6)** | **19K** | |
| **Goat-ScF14** | 19-21K | 526±46 (4/4) | 19-21K | **287±94 (4/4)** | **19-21K** | >650 (0/6) | - | **NA** | **NA** | NI ($Tg501F_{19\text{-}21K}$ / $Tg110F_{19K}$) |
| **Goat-Sc UKA2** | 21K | 245±36 (5/5) | 19-21K | **252±8 (6/6)** | **19-21K** | 255±69 (5/5) | 19K | **187±6 (6/6)** | **19K** | |
| **Goat-Sc UKB2** | 19-21K | 381 (1/5) | 19-21K | **214±34 (7/7)** | **19-21K** | 205±12 (7/7) | 19K | **196±5 (6/6)** | **19K** | |
| **Goat-Sc F10** | 21K | 449±19 (5/5) | 21K | **372±14 (6/6)** | **21K** | 556±68 (4/4) | 19K | **575±43 (5/5)** | **19K** | NI ($Tg501S_{21K}$ / $Tg110S_{19K}$) |
| **Goat-ScF11** | 21K | >650 (6/6) | 21K | **>330 (ongoing)** | **21K** | >650 (0/6) | - | **NA** | **NA** | |
| **Goat-ScG2** | 21K | >650 (1/4) | 21K | **>400 (ongoing)** | **21K** | 610 (1/6) | 19K | **348±85 (6/6)** | **19K** | |
| **Goat-ScG3** | 21K | 466±35 (4/4) | 21K | **375±25 (4/4)** | **21K** | >650 (0/6) | - | **NA** | - | |
| **Goat-Sc N1** | 21K | 535±8 (5/5) | 21K | **339±19 (5/5)** | **21K** | 649±9 (3/3) | 19K | **>450 (ongoing)** | - | |
| **Goat-Sc N2** | 21K | 555±4 (5/5) | 21K | **>350 (ongoing)** | - | >650 (0/13) | - | **NA** | - | |
| **Goat-Sc UKD2** | 21K | 547±100(5/5) | 21K | **>450 (ongoing)** | - | >650 (0/7) | - | **NA** | **NA** | |
| **Goat-Sc Zyp13** | 21K | 483±15 (4/4) | 21K | **301±10 (4/4)** | **21K** | 650 (2/2) | 19K | **371±51 (6/6)** | **19K** | |
| **Goat-Sc Zyp21** | 21K | 475±31 (5/5) | 21K | **324±9 (4/4)** | **21K** | >650 (2/6) | 19K | **387±12 (3/3)** | **19K** | |

[a] Brain $PrP^{res}$ was detected by Western blot using Sha31 (Fig. 14–16). The apparent molecular mass of the $PrP^{res}$ unglycosilated band is given in kilodaltons (K).

[b] n/n0: diseased, $PrP^{res}$: positive/inoculated animals.

[c] Isolates were considered fast (in red) if survival times were <300 days post-inoculation (dpi) at second passage, or slow (in blue) when survival times were >300 dpi at second passage.

* Among the Goat-Sc N3 infected BoPrP-Tg110 mice one exhibited very long survival times of 703 dpi. The brain from this animal was further passaged in BoPrP-Tg110 mice (in blue) separately from the other mouse (in red).

I: Italian
NI: Non-Italian
F: fast
S: slow
VS: very slow
NA: Not analyzed

Apart from the Italian isolates, Goat-scrapie F16 was the only isolate that exhibited a brain $PrP^{res}$ 21K unglycosilated band in the BoPrP-Tg110 mice. Nevertheless, unlike the "Italian" isolates, this isolate was transmitted to goat Wt-Tg501 mice as a fast isolate with a high transmission barrier (Table 24). These results suggest that Goat-Sc F16 could contain more than one strain that selectively replicates in terms of the $PrP^{C}$ species context (goat or bovine). As in the Conformational Selection model (Beringue et al., 2008b; Collinge and Clarke, 2007), the degree of overlap between the strains that the prion agent harbors and the conformational constraints imposed by the host $PrP^{C}$ (goat Wt-Tg501 vs. BoPrP-Tg110 mice) determine which strain is replicated.

The rest of the isolates (non-Italians) transmitted to the BoPrP-Tg110 mice can be separated into two groups according to the ST after second passage: "non-Italian fast" isolates (<300 dpi after second passage) or "non-Italian slow" isolates (>300 dpi after second passage) (Table 24). A number of "non-Italian" isolates were not transmitted in these mice, probably as a consequence of the "slow" condition previously described in Wt-Tg501 mice for these isolates in combination with a low infectious titer. For this reason, these isolates were included in the group of "non-Italian slow" isolates (Table 24).

The distribution of the two groups of "non-Italian isolates" ("fast" and "slow") overlaps considerably with that proposed for the Wt-Tg501 mice. Interestingly, an unexpectedly long ST in one BoPrP-Tg110 mouse infected with Goat-Sc N3 isolate (Table 24) indicates the possible co-existence of two different agents (a "non-Italian fast" plus a "non-Italian slow") in this isolate. This assumption was confirmed by further passages of this mouse brain that were undertaken separately from the rest of the mice with shorter ST (Table 24). Nevertheless, the question remains as to whether the Goat-Sc N3 as Goat-Sc F16 isolates originally contained more than one scrapie agent or whether new agents emerged after a mutation process occurring upon crossing the transmission barrier of BoPrP-Tg110 mice. Indeed, transmission of goat or sheep scrapie isolates in bovinized mice involves a species barrier that may result in the generation of new strain phenotypes (Beringue et al., 2008b; Collinge and

Clarke, 2007). Therefore, the serial passages of scrapie isolates in BoPrP-Tg110 mice might not reflect the original properties of the agent. The fact that different scrapie phenotypes can emerge from the same original isolate upon serial transmission in different mouse lines or animal species (Beck et al., 2012; Thackray et al., 2011; Thackray et al., 2008) highlights the need to use in combination a number of different animal models to characterize in detail the properties of scrapie strains.

Transmission studies of atypical scrapie isolates in both Wt-Tg501 and BoPrP-Tg110 models revealed a different scenario to classical scrapie isolates. All atypical scrapie isolates transmitted readily at first passage in Wt-Tg501 mice and had a 100% AR (Table 11), thereby confirming the potential for the natural transmission previously proposed for this atypical prion agent (Simmons et al., 2007; Simmons et al., 2011). In contrast to classical scrapie transmissions, atypical isolates consistently produced long ST (≈500–600 dpi), (Table 11), despite the high level of expression (2X) of the homozygous Wt-Tg501 mice used in this experiment. These results suggest that atypical scrapie is a slow infectious agent in their natural $PrP^{C}$ species context, which is consistent with the old age of naturally affected goats and sheep (Benestad et al., 2008).

In goat Wt-Tg501 mice, all the different atypical scrapie isolates exhibited indistinguishable $PrP^{res}$ WB glycoprofiles (Fig. 16B). Moreover, very similar transmission features were observed within atypical scrapie-infected BoPrP-Tg110 mice. Two different atypical scrapie isolates were able to infect these mice and a molecular shift to a BSE-like $PrP^{res}$ glycoprofile was observed in all infected brains (Fig. 15B).

Although histopathological analysis are not yet available, the homogeneity of the transmission results at first passage in both Wt-Tg501 and BoPrP-Tg110 mice supports the view that atypical scrapie isolates constitute a single prion agent (Gotte et al., 2011; Griffiths et al., 2010; Le Dur et al., 2005; Pirisinu et al., 2010; Pirisinu et al., 2013).

Wt-Tg501 mice were fully susceptible to the tested BSE isolates (Table 11) irrespective of their $PrP^{Sc}$ primary sequence (goat, sheep, or cattle). Goat-BSE isolates displayed similar ST to Cattle-BSE isolate at primary passage; no significant reduction in ST was observed for Cattle-BSE at second passage in goat Wt-Tg501 mice, thereby confirming the low transmission barrier of goats to cattle-BSE previously reported in experimentally challenged goats (Foster et al., 1993). Moreover, $PrP^{res}$ WB profile (Fig. 16) and histopathological alterations (Fig. 18A and B) in both goat-BSE- and cattle-BSE-infected Wt-Tg501 mice brains were similar in many ways.

By contrast, atypical Cattle-BSE L displayed an apparent transmission barrier in Wt-Tg501 mice that was similar to the one previously observed in ovinized TgOvPrP4 and Tg338 mice (Baron et al., 2007; Beringue et al., 2007; Nicot et al., 2013). Both 100% AR and very long ST were registered at first passage in Cattle-BSE L-infected Wt-Tg501 mice (Table 11). ST significantly decreased at second passage and was even shorter than those in cattle-BSE. Interestingly, atypical BSE L acquired a classical BSE-like $PrP^{res}$ WB profile in Wt-Tg501 mice (Fig. 16C) consistent with the phenotype shift of this agent in ovine-PrP Tg338 mice (Beringue et al., 2007). However, histopathological outcomes in atypical BSE L-infected mice clearly differed from those of classical BSE-infected animals at first passage (Fig. 18). Histopathological analysis of second passages are still ongoing aimed at determining whether atypical Cattle-BSE L acquires a classical BSE strain-like profile in further passages in goat Wt-Tg501 mice, as has been proposed by some authors (Beringue et al., 2007), or whether it maintains its specific strain properties as stated by others (Baron et al., 2007; Nicot et al., 2013).

It is important to note that some classical scrapie isolates showed an apparent transmission barrier when inoculated in goat Wt-Tg501 mice (Table 11). The origin of this barrier could be the presence of certain polymorphic variants in the $PrP^{Sc}$ sequence of some isolates that could have affected transmission efficiency at first passage. However, low infectious titers appear to be a more

likely cause of the limited transmissions observed at first passage since some of these polymorphic variants were harbored by other isolates that produced no transmission barriers (in orange in Table 11).

The effects of the PrP amino acid differences between hosts and donors in prion transmission and disease phenotype have been reported on numerous occasions for different combinations of prion strain-species (Barron et al., 2001; Manson et al., 1999). Intriguingly, although the histopathological analysis indicates the existence of distinct scrapie disease phenotypes (Fig. 17) among the scrapie-challenged Wt-Tg501 mice, the presence of polymorphisms in the amino acid sequence of the scrapie isolates did not seem to play any decisive role at this point. That is to say, Goat-Sc I3 and Goat-Sc I9, both classified as "Italian" isolates, were clearly different in their amino acid substitution at codon 143 ($R/H_{143}$) but exhibited no major differences in their neurological lesions (Fig. 17A) or $PrP^{Sc}$ distribution patterns (Fig. 16B). On the other hand, Goat-Sc F2 ("non-Italian fast" strain) and Goat-Sc F10 ("non-Italian slow" strain), both harboring the same $PrP^{Sc}$ amino acid sequence, clearly differed in their vacuolation scores when infecting Wt-Tg501 mice (Fig. 17B). Taken together, these results support the view that prion transmission features in Wt-Tg501 mice are mainly determined by the strain properties of the agent, while PrP amino acid sequence differences between hosts and donors only play a relatively minor role. In line with this view, atypical Cattle-BSE L showed a clear transmission barrier in goat Wt-Tg501, whereas classical Cattle-BSE, harboring the same $PrP^{Sc}$ sequence as Cattle-BSE L, was transmitted in this goat context without any apparent transmission barrier. As previously proposed by our group (Torres et al., 2014), these results recognize the pivotal role of the strain properties of a prion agent in the efficiency of its transmission to different species.

Taken together, all the scrapie and BSE transmission studies in Wt-Tg501 mice indicate that this mouse line is a highly valuable model i) for studying the susceptibility of goats and sheep to different scrapie and BSE strains and ii) for

identifying the scrapie strains affecting sheep and goat given that it reproduces their biological and biochemical properties. However, transmission studies in other animal models such as the BoPrP-Tg110 are also useful in understanding the variety of scrapie strains that are currently circulating in Europe and the mechanisms through which they are selected and/or adapted during transmission between species or even PrP genotypes.

**2. Analysis of the individual role of certain goat and sheep $PrP^C$ polymorphic variants in the susceptibility/resistance to prion infection by transmission studies in Tg mice.**

Amino acid differences between host $PrP^C$ and infectious $PrP^{Sc}$ can directly determine the rate of conversion of $PrP^C$ into $PrP^{Sc}$ and so reflect the transmissibility of prion agents and the susceptibility of hosts to prion infection (Bossers et al., 1997; Bossers et al., 2000). Interestingly, some polymorphic variants have no effect on this process, which thus suggests that not all variants influence prion conversion or prion replication in the same way.

To address this question, we produced transgenic (Tg) mice expressing different $PrP^C$ polymorphic variants (I/$M_{142}$, R/$H_{154}$, R/$Q_{211}$, Q/$K_{222}$, Q/$R_{171}$, or N/$K_{176}$) naturally found in sheep and/or goat species that vary only by a single amino acid at the corresponding PrP residue. $M_{142}$-Tg541, $H_{154}$-Tg563, $Q_{211}$-Tg580, $K_{222}$-Tg516, $R_{171}$-Tg552, and $K_{176}$-Tg570 mouse lines were IC inoculated with a variety of scrapie and BSE isolates previously used in the Wt-Tg501 mice challenges. The relative susceptibilities of these Tg mouse lines were compared with those obtained in Wt-Tg501 mice to determine the individual role of each polymorphic variant in the susceptibility/resistance to prion infection.

$K_{222}$-Tg516 mice expressing the $K_{222}$-$PrP^C$ variant were fairly resistant to all the inoculated classical scrapie isolates (Table 12), regardless of their geographical origin, the polymorphic variants originally in their $PrP^{Sc}$ sequences, their $PrP^{res}$ WB profile, or their behavior upon passage in both BoPrP-Tg110 (Table 10) and Wt-Tg501 mice (Table 11). No $PrP^{res}$ or histological alterations were observed in any of the brains of these challenged $K_{222}$-Tg516 mice and only some vacuolation – similar to that of non-inoculated aged mice – was detected (Fig.

20). Since both Wt-Tg501 and $K_{222}$-Tg516 mice express exactly the same $PrP^C$ sequence (except for the Q/$K_{222}$ amino acid substitution) and have similar $PrP^C$ expression levels, we can assume that the lack of transmission efficiency for classical scrapie in $K_{222}$-Tg516 mice is linked to the Q/$K_{222}$ polymorphism. This conclusion is consistent with previous epidemiological studies carried out in numerous European goat scrapie outbreaks in which the $K_{222}$-$PrP^C$ variant was only detected in healthy goats (Acin et al., 2013; Acutis et al., 2006; Barillet et al., 2009; Kanata et al., 2014; Vaccari et al., 2006). Taken together, these results suggest that the $K_{222}$ variant of goat $PrP^C$ is strongly resistant to a wide range of classical scrapie isolates. Intriguingly, atypical scrapie isolate Sheep-M45 infected 100% $K_{222}$-Tg516 mice with similar ST (Table 12), $PrP^{res}$ WB glycoprofile (Fig. 19), and histopathological alterations (Fig. 20) than those observed in Wt-Tg501 mice. These results suggest that, despite its high resistance to classical scrapie, the $K_{222}$-$PrP^C$ variant confers no resistance to the replication of the atypical scrapie agent.

BSE transmission experiments showed that $K_{222}$-Tg516 mice were resistant to classical Cattle-BSE and atypical Cattle-BSE L but not to goat-BSE or sheep-BSE isolates (Table 12). The failure of the cattle-BSE isolate to be transmitted to $K_{222}$-$PrP^C$ mice can be explained by the dual influence of the $PrP^C$ sequence differences in prion cross-species transmission (Agrimi et al., 2003; Baylis et al., 2004; Torres et al., 2014) and the effect of the Q/$K_{222}$ amino acid substitution. In this sense, the effect of the $PrP^C$ primary sequence of the donor (bovine) is not strong enough to avoid cattle-BSE replication in goat Wt-Tg501 mice (Table 9). However, after the Q/$K_{222}$ substitution in $K_{222}$-Tg516 mice, the cattle-BSE agent is unable to replicate. This view is fully consistent with the fact that $K_{222}$-Tg516 mice were susceptible to BSE only after passage in sheep, goat, or Wt-Tg501 mice (Table 12). Therefore, the transmission barrier toward the BSE agent is complex and is modulated not only by the $K_{222}$ allele but also by other determinants such as the PrP amino acid differences between hosts and donors (goats vs. cattle).

Taken together these transmission experiments demonstrate that the single amino acid exchange from a glutamine to a histidine at codon 222 ($Q/K_{222}$) provides resistance to infection with classical scrapie, classical cattle BSE, and atypical cattle BSE L but not to atypical scrapie, sheep BSE, or goat BSE. Most of these results, together with some transmission outcomes in the goat Wt-Tg501 mouse line, have been published recently (Aguilar-Calvo et al., 2014).

$Q_{211}$-Tg580 mice expressing the $Q_{211}$-$PrP^C$ variant were resistant to all the inoculated classical scrapie isolates (Table 13). Scrapie-challenged $Q_{211}$-Tg580 mice showed no neurological sign of prion disease or $PrP^{Sc}$ in their brains after analysis by WB or PET-blot (Table 13), thereby suggesting that, like the $K_{222}$ variant, the $Q_{211}$-$PrP^C$ variant confers high resistance to classical scrapie infection. This assumption is consistent with the decreased susceptibility to natural infection with classical scrapie associated with $R/Q_{211}$ goats (Barillet et al., 2009; Bouzalas et al., 2010). However, unlike the $K_{222}$-$PrP^C$ variant, natural scrapie infection in $R/Q_{211}$ goats has been more often reported than in $Q/K_{222}$ goats (Acutis et al., 2006; Barillet et al., 2009; Fragkiadaki et al., 2011), which indicates that the $K_{222}$-$PrP^C$ variant would provide a greater degree of resistance to classical scrapie than the $Q_{211}$-$PrP^C$ variant. Accordingly, cell-free conversion studies have shown that the conversion capacity of the $K_{222}$ allele with scrapie is completely abolished, while the $Q_{211}$ allele only has a lower conversion capacity than the goat WT allele (Eiden et al., 2011).

The atypical scrapie transmission studies revealed that $Q_{211}$-Tg580 mice were susceptible to this prion agent, albeit with very long ST (>650 dpi) compared to homozygous Wt-Tg501 mice (Table 13). These prolonged ST could be associated with the decreased susceptibility to atypical scrapie provided by the $Q_{211}$-$PrP^C$ variant; nevertheless, lower $PrP^C$ levels of expression in the brain of $Q_{211}$-Tg580 mice (1X) than homozygous Wt-Tg501 mice (2X) could also have caused these contrasting results. Finally, no $Q_{211}$-Tg580 mice have yet succumbed to the

infection with any of the BSE isolates inoculated. Experiments are ongoing to conclude at this point.

All in all, these transmission experiments with $Q_{211}$-Tg580 mice suggest that the arginine-to-glutamine amino acid substitution at codon 211 ($R/Q_{211}$) leaves the mutated $PrP^C$ unable to replicate any classical scrapie agent but has any substantial effect in the susceptibility to the infection with atypical scrapie.

Despite the low levels of $PrP^C$ expressed in their brains (0.25X), $H_{154}$-Tg563 mice were susceptible to some scrapie and BSE isolates (Table 14). These positive transmissions suggest a low or null effect of the $H_{154}$-$PrP^C$ variant in susceptibility/resistance to scrapie and BSE infection. In line with this view, homozygous $H/H_{154}$ sheep and goats were reported to be susceptible to both scrapie (Luhken et al., 2004) and BSE infection (Foster et al., 2001a). A Tg mouse line expressing higher levels of brain $H_{15}$-$PrP^C$ would be required to be able to draw conclusions regarding the role of this polymorphic variant in susceptibility to prion infection.

$M_{142}$-Tg541 mice were susceptible to most of the inoculated classical scrapie isolates despite displaying statistically significant longer ST than Wt-Tg501 mice in all the cases. These delays in ST agree with the decreased susceptibility to scrapie associated with $I/M_{142}$ polymorphism in several naturally infected goat herds (Acin et al., 2013; Barillet et al., 2009; Goldmann et al., 2011; Gonzalez et al., 2010), as well as in experimental transmission studies (Goldmann et al., 1996), but also support the contention that the effect of $M_{142}$ $PrP^C$ polymorphic variant is limited to prolonging survival times (ST). Curiously, the delays in ST observed in scrapie-infected $M_{142}$-Tg541 mice were significantly higher in "non-Italian fast" isolates (Goat-Sc F2, Goat-Sc S3, and Sheep-Sc Langlade) than in "non-Italian slow" isolates (Goat-Sc F10 and Goat-Sc S2), whilst "Italian isolates" (Goat-Sc I3 and Goat-Sc I9) were not transmissible in $M_{142}$-Tg541 mice (Table 13). These results suggest a strain-dependent effect for $M_{142}$-$PrP^C$ in infection with classical scrapie agents. The failure of "Italian" isolates to replicate in $M_{142}$-Tg541 mice could result from the

combination of the slow velocity of replication of these isolates (Table 15) and the effect of long ST provided by the $M_{142}$-$PrP^C$ variant. Further passages are currently in progress aimed at tackling these questions and discarding a possible strain-dependent resistance of this polymorphic variant to "Italian" scrapies.

Unlike the scrapie challenges, no effect of the I/$M_{142}$ polymorphism could be observed in the BSE transmissions in $M_{142}$-Tg541 mice (Table 15). These animals succumbed to all the BSE isolates inoculated and showed very similar ST than Wt-Tg501 mice, which contradicts the lower susceptibility to BSE attributed to the I/$M_{142}$ polymorphism in previous experimental inoculations in goats (Goldmann et al., 1996). The influence of other genetic factors besides *prnp* genotype could have biased the results obtained in the goat bioassay in which the animals were of a genetic mixture (Goldmann et al., 1996). More interestingly, none of the $M_{142}$-Tg541 mice showed any sign of neurological disease when challenged with atypical scrapie isolates (Table 15) and no $PrP^{res}$ could be detected in their brains by WB analysis. These results suggest that the I/M142 polymorphism might play a determinant role in susceptibility to atypical scrapie infection. To our knowledge, this is the first time that a goat $PrP^C$ variant has been associated with greater resistance to atypical scrapie, a finding that may have important implications in the control of this disease in goat herds.

These transmission experiments in $M_{142}$-Tg541 mice demonstrate that the single isoleucine-to-methionine amino acid exchange at codon 142 (I/$M_{142}$) affects the replication with classical and atypical scrapie agents in a strain-dependent manner; but has any substantial effect on BSE replication.

$R_{171}$-Tg552 mice, expressing the $R_{171}$-$PrP^C$ variant, were resistant to all the scrapie isolates inoculated. These animals showed no clinical signs of prion disease and neither $PrP^{res}$ was detected in their brains (Table 16). These results are consistent with the high resistance of Q/$R_{171}$ polymorphism to prion infection obtained in numerous epidemiological and experimental studies in

sheep and goats (Bouzalas et al., 2010; Gonzalez et al., 2007; Gonzalez et al., 2005; Greenlee et al., 2014) and the low number of naturally infected sheep or goats harboring this polymorphism (Groschup et al., 2007; Ikeda et al., 1995) and are the basis of the selection of the $R_{171}$-$PrP^C$ variant in selective breeding programs against scrapie.

By contrast, $R_{171}$-Tg552 mice were susceptible to the infection with atypical scrapie (Table 16). The susceptibility of ARR sheep to atypical scrapie is well known; however we could not determine an increased susceptibility of the $R_{171}$-$PrP^C$ variant to atypical scrapie, as proposed by epidemiological studies (Benestad et al., 2008). BSE transmission studies in $R_{171}$-Tg552 mice are still ongoing. Though ST of these mice surpass those of Wt-Tg501 mice, we cannot draw final conclusions regarding the effect of the $R_{171}$-$PrP^C$ variant in the susceptibility to BSE infection.

Hence, transmission studies in $R_{171}$-Tg552 mice demonstrate that the single amino acid exchange glutamine-to-arginine at codon 171 ($Q/R_{171}$) confers resistance to infection with classical scrapie but not with atypical scrapie.

Transmission studies in $K_{176}$-Tg570 mice produced highly promising results. None of the $K_{176}$-Tg570 mice succumbed to the primary transmission of any inoculated classical scrapie isolates (Table 17). No $PrP^{res}$ or neuropathological alterations were observed in these mice. The $K_{176}$-$PrP^C$ variant has previously been linked to a high level of protection against scrapie in epidemiological studies (Maestrale et al., 2009; Vaccari et al., 2009b) in sheep experimentally challenged with one scrapie isolate (Vaccari et al., 2007) and in PMCA studies (Bucalossi et al., 2011). Moreover, only one scrapie field case of a sheep harboring this *prnp* genotype in heterozygosis has ever been documented (Maestrale et al., 2009), which is evidence of the good resistance of the $K_{176}$-$PrP^C$ variant to classical scrapie. By contrast, $K_{176}$-Tg570 mice succumbed to atypical scrapie transmission and had even shorter ST than Wt-Tg501 mice (Table 17). These transmission results suggest that the $K_{176}$-$PrP^C$ variant could increase the capacity of the mutated $PrP^C$ to replicate atypical scrapie agents.

The decisive role of the $K_{176}$-$PrP^C$ variant against infection with classical BSE was successfully established during our transmission studies with Tg mice. $K_{176}$-Tg570 mice did not succumb to the inoculation with Cattle-BSE (Table 17) and their brains were scored $PrP^{res}$ negative by WB analysis. Consistent with our results is the increased resistance of the $K_{176}$-$PrP^C$ variant to the IC transmission of cattle-BSE that has previously been reported in sheep (Vaccari et al., 2007). Remarkably, our transmission studies in Tg mice permitted us to demonstrate the resistance of the $K_{176}$-$PrP^C$ variant to BSE even after being adapted to sheep and goat $PrP^C$ sequences (Table 17), as was previously observed in goat $Q_{211}$-Tg580 mice but not in $K_{222}$-Tg516 mice. Therefore, contrary to $Q/K_{222}$, the effect of the $N/K_{176}$ amino acid exchange is strong enough to block the transmission of the BSE agent without adding the effect of the $PrP^C$ sequence differences in prion cross-species transmission (bovine vs. ovine).

Taken together, these transmission results strongly support the view that the single amino acid asparagine-to-lysine substitution at codon 176 ($N/K_{176}$) triggers a high level of resistance to infection with classical scrapie and BSE. This affirmation is reinforced by the fact that $K_{176}$-Tg570 mice overexpress the $K_{176}$-$PrP^C$ variant (2-fold the $PrP^C$ expression in the sheep brain) and therefore a low $PrP^C$ expression in these mice cannot explain the resistance to these TSEs. These studies also indicate that the $N/K_{176}$ polymorphism might increase susceptibility to atypical scrapie infection. Results from prion transmission experiments in both $R_{171}$-Tg552 and $K_{176}$-Tg570 mouse lines will be included in two articles in preparation (see Publications section).

In summary, the use of this panel of transgenic mice, all with very similar genetic backgrounds and the same goat-wt $PrP^C$ but with a single amino acid substitutions, allowed us to identify certain amino acid substitutions as the sole cause of the differential susceptibility to prion infection, thereby excluding the possibility of any part played by other genetic factors. In addition, the intracerebral inoculation route provided the best scenario for prion replication since the inoculum was placed directly in the target tissue. Therefore, our

results offer solid arguments to support the hypothesis that certain variants of the $PrP^C$ strongly modulate the susceptibility of sheep and goats to classical scrapie, classical BSE, and/or atypical BSE L. These studies also indicate that the amino acid exchanges tested in this work have no major effect in the brain $PrP^{res}$ WB profile and in the histopathological outcomes.

Interestingly the effect of the $PrP^C$ polymorphic variant in susceptibility to prion infection was extremely affected by the prion agent in these studies (Table 25). This phenomenon was particularly remarkable in the case of scrapie infections. Therefore, the polymorphic variants associated with high resistance to classical scrapie ($K_{222}$-, $Q_{211}$-, $K_{171}$- and $K_{176}$-$PrP^C$ variants) were susceptible to atypical scrapie, whereas those that exhibited no or no major effect on susceptibility to classical scrapie infection ($M_{142}$-$PrP^C$ variant) appeared as strong modulators of susceptibility to atypical scrapie (Table 25), which indicates that there might be an opposite effect of polymorphic variants in susceptibility to classical and atypical scrapie infection. This assumption was previously proposed for sheep naturally infected with atypical scrapie. Hence, ARR sheep are more frequently affected by atypical scrapie disease than ARQ or VRQ sheep (Benestad et al., 2008). Nevertheless, more information is still required to understand the role of *prnp* genotype in the susceptibility to this agent.

**Table 25.** Summary of the results obtained during the transmission studies in the different goat and sheep PrP Tg mouse lines

| TSE | $M_{142}$-Tg541 | $H_{154}$-Tg563 | $K_{222}$-Tg516 | $Q_{211}$-Tg580 | $R_{171}$-Tg552 | $K_{176}$-Tg570 |
|---|---|---|---|---|---|---|
| **Classical scrapie** | Prolonged ST[a] | No effect* | **High resistance** | **High resistance** | **High resistance** | **High resistance** |
| **Atypical scrapie** | **High resistance** | NA | No effect | No effect | No effect | No effect |
| **Goat-BSE** | No effect | No effect* | No effect | Ongoing | Ongoing | **High resistance** |
| **Sheep-BSE** | | | | | | |
| **Cattle-BSE** | | | **High resistance** | | | |
| **Atypical BSE L** | NA | NA | **High resistance** | NA | Ongoing | Ongoing |

[a] Survival times.

* The low levels of $PrP^C$ expressed in the brain of $H_{154}$-Tg563 mice prevents from concluding on the effect of this $PrP^C$ variant in the susceptibility to prion infection.
NA: Not analyzed.

The molecular mechanisms through which $PrP^C$ polymorphic variants affect susceptibility to prion infection are not known. It has been proposed that mutations in the $PrP^C$ sequence may influence the capacity of the prion protein to bind $PrP^{Sc}$ (Eiden et al., 2011; Perrier et al., 2002). However, it has also been reported that scrapie-resistant variant $R_{171}$-$PrP^C$ binds to $PrP^{Sc}$ as efficiently as prion-susceptible variant $V_{136}$-$PrP^C$ and sheep wt-$PrP^C$ (Rigter and Bossers, 2005). In addition, *in vitro* experiments have shown that the $PrP^C$ of more than one species (hamster and mouse) binds equally efficiently to mouse $PrP^{Sc}$ but preserves its conversion specificity (Horiuchi et al., 2000). Therefore, a further step in the conversion process could decrease or inhibit prion conversion. This information provides clues for designing therapeutic strategies based on blocking $PrP^{Sc}$ through binding prion-resistant $PrP^C$ variants (see below).

An effect of $PrP^C$ variants on the thermodynamic stability and $PrP^C$-folding kinetics of the prion protein has been previously reported (Eghiaian et al., 2004; Fitzmaurice et al., 2008; Paludi et al., 2007; Rezaei et al., 2002). Indeed, the amino acid exchange from a glutamine to an arginine at codon 171 ($Q/R_{171}$) has been reported to cause the destabilization of the prion protein conformation (Eghiaian et al., 2004) (Fig. 31) and to be associated with increased protease sensitivity and decreased formation of large amyloid fibrils (lower amyloidogenesis) (Rezaei et al., 2002). $A/V_{136}$ sheep polymorphism also affects $PrP^C$ stability since it is close to the region that is supposedly involved in refolding $PrP^C$ to $PrP^{Sc}$ (residues 129–134 and 163–167 of the sheep sequence) (Eghiaian et al., 2004; Rezaei et al., 2002), while the $R/H_{154}$ amino acid exchange alters the α-helix 1 of the sheep $PrP^C$ (Megy et al., 2004). These findings suggest the need for a structural analysis of our $PrP^C$ variants as a means of addressing the molecular basis of their variable role in susceptibility/resistance to the infection with different prion strains.

### 3 Protective effect of the $K_{222}$ polymorphic variant of goat $PrP^C$ on prion replication of the goat-wild type allele

In order to study the hypothetical inhibitory influence of the $K_{222}$-$PrP^C$ variant on goat wt $PrP^C$, heterozygous Q/$K_{222}$ mice were obtained and IC-challenged with a variety of scrapie and BSE isolates.

Heterozygous Q/$K_{222}$ mice were resistant to most of the classical scrapie isolates and, once they succumbed to the infection, had very long ST and lower AR than Wt-Tg501 mice (Table 18). These results support the contention that the $K_{222}$-$PrP^C$ variant has a dominant negative effect over the wild type $PrP^C$ sequence. This phenomenon is consistent with the stone-fence model (Kobayashi et al., 2009) that predicts that, for a given TSE agent, the incorporation of a conversion-incompetent $PrP^C$ variant ($K_{222}$) will interfere with wild type $PrP^{Sc}$ replication, thereby resulting in less efficient prion propagation. This decreased propagation efficiency would lead to lower AR and/or prolonged ST – as shown in our study – and could explain the lack of epidemiological evidence linking scrapie with the $K_{222}$ allele (Barillet et al., 2009; Fragkiadaki et al., 2011; Jeffrey et al., 2014; Papasavva-Stylianou et al., 2011). Interestingly, the previously determined transmission barrier of $K_{222}$-Tg516 mice to Cattle-BSE was abolished when the $K_{222}$-$PrP^C$ variant was found in heterozygosis (Table 18), which indicates that for classical BSE Q/$K_{222}$ amino acid substitution does not interfere with the conversion of wt $Q_{222}$-$PrP^C$ into $PrP^{Sc}$.

The question remains, though, whether the wt allele or the $K_{222}$-$PrP^C$ variant is responsible for prion replication in scrapie and BSE-infected Q/$K_{222}$ mice (Table 25). The existence of a monoclonal antibody (mAb) able to selectively target the $K_{222}$ epitope instead of $Q_{222}$ would help solve this question. Unfortunately, this Ab does not exist and so further passages of scrapie and BSE adapted to heterozygous Q/$K_{222}$ mice in both Wt-Tg501 and $K_{222}$-Tg516 mice (Table 19) were necessary. The positive transmission observed in Wt-Tg501 mice together with the resistance of $K_{222}$-Tg516 mice to this inoculation allow us to conclude that goat wt allele ($Q_{222}$) is the only allele replicating the prion agent in Q/$K_{222}$

heterozygous mice, at least for the Goat-Sc F2 isolate. However, the absolute inability of $K_{222}$ allele to replicate scrapie can be ruled out if we bear in mind our transmission studies in goats in which one homozygous $K/K_{222}$ goat was infected by the IC route with one classical scrapie case (Table 20). A reason for this discrepancy could be the shorter lifespan of mice than goats, which could hinder the development of the prion disease or the scrapie strain. Thus, further studies are still needed to provide an answer to this question.

This dominant negative effect provided by the goat $K_{222}$-$PrP^C$ variant against the scrapie replication has previously been described by cell-free conversion analysis (Eiden et al., 2011). Furthermore, an inhibitory effect on the prion replication of equivalent $K_{222}$ variants in other species has also been described. Human $E/K_{219}$ polymorphism (homologous to the $Q/K_{222}$ polymorphic variant of the goat $PrP^C$) has been linked to protecting humans against sporadic Creutzfeldt-Jakob-Disease in epidemiological studies in Japanese (Shibuya et al., 1998) and other Asiatic (Soldevila et al., 2003) populations. Moreover, KI Tg mice expressing the $E/K_{219}$ polymorphism in heterozygosis were resistant to sCJD (Hizume et al., 2009). Similarly, the equivalent $K_{218}$ variant of the mouse $PrP^C$ significantly reduced the fibril aggregation kinetics and generated non-proteinase K-resistant PrP in fibrillization experiments with recombinant $Q/K_{218}$ (Lee et al., 2007). The dominant-negative inhibition provided by this polymorphism has also been demonstrated in scrapie-infected neuroblastoma cells (Crozet et al., 2004; Kaneko et al., 1997), as well as by PMCA (Geoghegan et al., 2009).

The molecular mechanism driving this inhibitory effect is poorly known. A plausible reason could be the insertion of an additional positive charge at codon 222, 219, or 218 (of the goat, human, and mouse PrP sequences, respectively) provided by the lysine amino acid (K), which would interfere with the $PrP^C/PrP^{Sc}$ interaction and lead to abolished or low conversion rates of $PrP^{Sc}$ (Eiden et al., 2011). In this sense, comparative studies of the NMR structures of human recombinant prion protein harboring either the wt genotype or the $E/K_{219}$ polymorphism have linked the protective effect of this polymorphism to

perturbations in surface charge distribution and structural rearrangements (mainly localized at the β2-α2 loop region) (Biljan et al., 2012). More recently, molecular dynamic studies comparing human dimeric homozygous Wt PrP-Wt PrP forms to heterozygous Wt PrP-$K_{219}$ PrP forms suggest that the dominant-negative effect of this protective mutation of this polymorphism ($E/K_{219}$) is due to the incompatible structures and dynamics of allelic variants during the conversion process (Jahandideh et al., 2014).

Similarly, the resistance provided by the $K_{222}$-$PrP^C$ variant in our Tg mice could depend on the conformational changes derived from the Q-to-K amino acid substitution at codon 222. Therefore, comparative studies on the conformational stability of $Q_{222}$ and $K_{222}$ prion proteins will help understand the molecular basis of the selective resistance of goat $Q/K_{222}$ polymorphic variant to classical scrapie and cattle-BSE but not to atypical scrapie or goat or sheep-BSE. Understanding these phenomena would finally help us to design therapeutic strategies based on blocking prion replication.

**4. Effect of polymorphic variants of the $PrP^C$ on susceptibility to prion infection in the natural host (goats)**

Our results in Tg mice models offer solid arguments supporting the view that certain variants of the $PrP^C$ strongly modulate the susceptibility of sheep and goats to prion infection. However, our procedure neither mimics the natural route of scrapie infection nor the complex pathogenesis involving prion replication in peripheral tissues, thereby highlighting the necessity to confirm this outcome in a natural host. Thus, to study the reliability of the results obtained in Tg mice, we worked in collaboration with two other research groups, with whom we performed the IC and/or oral inoculations with a classical scrapie isolate and a goat-BSE isolate in different PrP genotype goats.

Experimental transmissions of a scrapie field case by either the oral or the IC route to goats clearly reveal the effect of $PrP^C$ variants on the susceptibility of this species to classical scrapie. This goat bioassay, performed in the ENVT (Toulouse) within the framework of a collaboration with Dr. Andreoletti´s group, showed that goats harboring the $M_{142}$-$PrP^C$ variant were fully

susceptible to both the oral and the IC inoculation of the scrapie isolate, albeit with much longer survival periods than WT PrP goats (Table 20). These results agree with the delays in ST registered in scrapie-infected $M_{142}$-Tg541 mice (Table 15) and confirm that the $M_{142}$-$PrP^C$ variant does not play a decisive role in the susceptibility of goats to classical scrapie infection.

$H_{154}$ and $Q_{211}$ goats were resistant to oral inoculation in this scrapie field case (Table 20). However, 100% AR was observed in animals bearing these genotypes after an IC challenge, thereby indicating that $H_{154}$ and $Q_{211}$-$PrP^C$ variants have a strong protective effect against oral scrapie infection, which can be overcome by the IC route. Notably, IC-challenged $Q_{211}$ goats had three-times longer incubation periods than WT animals (Table 20), while IC-challenged $H_{154}$ goats had smaller delays in incubation periods than even $M_{142}$ goats (Table 20).

Most interestingly, the high resistance to classical scrapie associated with $K_{222}$-$PrP^C$ variant in our transmission studies in Tg mice was reinforced in the goat bioassay. None of the $K_{222}$ goats succumbed to the oral infection with the classical scrapie isolate and only a few succumbed to the IC infection, and all had very long survival times (four- to five-times the incubation periods in WT goats). Remarkably, of the goats that succumbed to the scrapie IC challenge, one was homozygous for the $K_{222}$ genotype, which shows that the $K_{222}$-$PrP^C$ variant is able to replicate at least one classical scrapie isolate and that the resistance provided by the $K_{222}$-$PrP^C$ variant is extremely high but not absolute.

In combination, these transmission experiments in goats confirmed most of the results obtained in the different Tg mouse lines and therefore were recently published (Lacroux et al., 2014a) along with the transmission studies in Tg mice (Aguilar-Calvo et al., 2014).

A genotype-dependent transmission of goat-BSE was demonstrated by goat bioassay at the FLV (Insel Riems). In this study, Goat-BSE-orally infected WT goats developed the first clinical signs of TSE at 24 mpi after inoculation (Table 21). Clinical signs were similar to previous BSE inoculations in sheep and goats (Foster et al., 2001a; van Keulen et al., 2008a). $PrP^{Sc}$ deposits in the brain and

neurodegenerative lesions also agreed with those previously observed in BSE-inoculated sheep and goats (Foster et al., 2001a; Gonzalez et al., 2005; Lezmi et al., 2011), and were mainly detected at the level of the obex. Additionally, prion infectivity was also present in muscles and LN tissues (detected by mouse bioassay), albeit with smaller titers than in the brain. These results are consistent with $PrP^{Sc}$ spread from the central nervous system (CNS) to peripheral tissues as reported in BSE oral transmission in sheep and cattle (Buschmann and Groschup, 2005; van Keulen et al., 2008b).

$R/Q_{211}$ goats first developed BSE at 33 mpi (Table 21). Prion infectivity in $R/Q_{211}$ goats was noticeably higher in the brain than in peripheral tissues, once again in agreement with the WT goat results (Table 23). Interestingly, for the $R/Q_{211}$ goats the onset of the disease was delayed by 9–12 months, which suggests the existence of a genotype-dependent transmission of BSE to goats in which the $Q_{211}$ $PrP^{C}$ polymorphic variant might have an effect on the incubation period but not on susceptibility. Therefore, $R/Q_{211}$ polymorphism could be associated with low resistance to goat-BSE, in contrast to the high resistance to scrapie associated with this polymorphism in field studies (Bouzalas et al., 2010; Corbiere et al., 2013b) and our experimental transmissions in goats. This discrepancy in the results regarding the $Q_{211}$ $PrP^{C}$ variant and TSEs transmission is likely also to be linked to the prion strain as a determinant factor in BSE and scrapie occurrence (Aguzzi et al., 2007), as revealed by our transmission studies in Tg mice (Table 13).

In contrast to the susceptibility of WT and $R/Q_{211}$ goats to goat-BSE, none of the $Q/K_{222}$ goats showed any evidence of disease (Table 21) and were culled at 86 mpi because of intercurrent diseases. No clinical signs or $PrP^{res}$ were observed in any of these $Q/K_{222}$ goats and histopathological analysis revealed no $PrP^{Sc}$ deposits or neurological lesions. However, low infectivity was detected in the brain of one of the two $Q/K_{222}$ goats euthanized at 44-45mpi (Table 23). Traces of infectivity were also detected in psoas major muscles from both goats, thereby suggesting that the $K_{222}$ *prnp* variant drastically decreases the susceptibility of goats to goat-BSE. In fact, transmission studies in Tg mice have

shown that $K_{222}$-Tg516 mice expressing the goat $K_{222}$-$PrP^C$ variant were 100% susceptible to the IC transmission of goat-BSE and had similar ST to goat wt-PrP transgenic mice (Table 12). This result suggests that the $K_{222}$-$PrP^C$ variant is as able to replicate the goat-adapted BSE as the goat WT-$PrP^C$. Hence, the resistance to goat-BSE might not be an intrinsic molecular property of the $K_{222}$-$PrP^C$ variant and the low susceptibility of $Q/K_{222}$ goats to oral infection with goat-BSE might also be influenced by factors other than the host *prnp* genotype.

A plausible reason for the discrepancy between the results from goat-BSE transmissions to $Q/K_{222}$ goats and $K_{222}$-Tg516 mice could be the infection route. Intracerebral inoculation (performed for transmission studies in Tg mice) provides the best route for prion replication since the inoculum is directly placed in the target tissue. By contrast, in oral transmission (used for goat inoculation) prions must overcome a succession of obstacles if they are to reach the target tissues: i) the crossing of the mucosal barrier, ii) amplification in gut-associated lymphoid tissues, iii) lymphatic/haematogenic dissemination, iv) neuroinvasion via the peripheral nervous system (PNS), and, finally, v) reach the CNS (van Keulen et al., 2008a). One or more of these steps might be affected in some way by the *prnp* genotype and thus modulate the BSE capacity to replicate and/or spread through the organism.

In line with this view, some authors have proposed that the ability of peripherally injected prions to replicate in extra-neural tissues such as Peyer's patches in the intestinal tract, spleen, tonsils, appendix, and lymph nodes may be critical in determining whether or not prions can persist in the host before spreading to the PNS and then to the CNS (Beringue et al., 2008b). Lymphotropism seems to play an important role in prion infection. While BSE-$PrP^{Sc}$ in cattle is mainly confined to the CNS and the vegetative nervous system (Buschmann and Groschup, 2005; Kaatz et al., 2012), the BSE agent becomes lymphotropic in sheep and goats and a more widespread $PrP^{Sc}$ distribution including several lymphoid tissues (Bellworthy et al., 2005; Foster et al., 1996a; Foster et al., 2001b) has been observed in BSE-infected goats and sheep. In BSE oral infected sheep, a $PrP^{Sc}$ replication in certain lymphoid tissues such as the

tonsils and ileal Peyer's patches before the infection of some areas of the ENS has been reported (Andreoletti et al., 2000; Kujala et al., 2011). On this basis, we hypothesize that the resistance of Q/$K_{222}$ goats to oral goat-BSE infection could be linked to the poorer capacity of goat-BSE to convert $K_{222}$-$PrP^C$ in peripheral organs. Lower levels of expression of $K_{222}$-$PrP^C$, together with the regional variability of the $K_{222}$-$PrP^C$ isoforms in these organs of primary transmission, cannot be ruled out.

Discrepancies in prion transmission efficiency linked to the inoculation route have previously been described (see review in (Beringue et al., 2008c)). In our goat bioassay, $H_{154}$, $Q_{211}$, and $K_{222}$ goats were completely resistant to scrapie inoculation via the oral route but susceptible via the intracerebral route (Table 20). Interestingly, while 100% of the $H_{154}$ and $Q_{211}$ goats succumbed to the scrapie IC inoculation, only few $K_{222}$ goats developed the disease with 4–5 times longer incubation periods than WT goats, which supports the view that the $K_{222}$ variant renders goats less susceptible to scrapie infection than the $Q_{211}$ variant. This statement could be extended to BSE infection, as supported by our transmission experiments of goat-BSE to goats.

Alternatively, different levels of the C1 fragment in $K_{222}$-$PrP^C$ could exist. For sheep, the proteolytic processing of its $PrP^C$ seems to be *prnp* genotype-dependent. Hence, more of the C1 fragment and less of the C2 fragment were reported in brains from sheep linked to resistance to scrapie (Campbell et al., 2013). Indeed, the recombinant protein comprising the C1 fragment of the ovine ARR variant was reported to delay or even inhibit the fibrillization of full length PrP (Campbell et al., 2013). Similarly, the proteolytic processing of the $K_{222}$-$PrP^C$ variant could produce greater amounts of C1 fragment than goat wt-$PrP^C$, thereby blocking or decreasing the aggregation of this polymorphic variant.

Finally, it is important to note that heterozygous Q/$K_{222}$ goats were considerably less susceptible to the transmission of goat-BSE than WT goats (Table 21), indicating that the $K_{222}$ $PrP^C$ variant could also protect against goat-BSE oral infection. Nevertheless, since homozygous K/$K_{222}$ goats were not

challenged in this study, we cannot draw any conclusions regarding the level of resistance provided by this variant or its ability to replicate goat-BSE.

The results obtained in Goat-BSE oral transmission to goats demonstrate genotype-dependent transmission in which the $K_{222}$-$PrP^C$ variant plays a decisive role. The protective effect provided by the $K_{222}$-$PrP^C$ variant against goat-BSE (results included in a recently submitted article; see Publications section) complement well its protective effect against scrapie, previously reported in goats and Tg mice. This finding further confirms the ability of the $K_{222}$-$PrP^C$ variant to control the horizontal transmission of both classical scrapie and goat-BSE diseases in goat herds through selective breeding programs.

# VIII. CONCLUSIONS

1. Wt-Tg501 mouse line is a highly valuable model for studying the susceptibility of goats and sheep to different scrapie and BSE strains and for identifying the scrapie strains affecting sheep and goat. Transmission studies in other animal models such as the BoPrP-Tg110 are also useful in understanding the prion strain diversity.
2. Transmission experiments in both Wt-Tg501 and BoPrP-Tg110 mice show the existence of at least four classical scrapie strains circulating in Europe and exhibiting a regional distribution. By contrast, atypical scrapie appears to be a homogeneous disease –unique strain- with the potential for natural transmission in both the goat- and bovine-PrP context.
3. Goat Wt-Tg501 mice showed low or null transmission barrier to classical cattle-BSE but moderate to high transmission barrier towards atypical cattle-BSE L which highlights the effect of the strain in the bovine-goat species barrier.
4. Transmission studies in Tg mice expressing goat and sheep $PrP^C$ polymorphic variants allowed us to conclude that the single glutamine-to-lysine amino acid substitution at codon 222 ($Q/K_{222}$) provides resistance to infection with classical scrapie, classical cattle BSE, and atypical cattle BSE L but not to atypical scrapie, sheep BSE, or goat BSE. Moreover, the $K_{222}$-$PrP^C$ variant displays a dominant negative effect over the wild-type $PrP^C$ sequence which results in a lower efficacy of classical scrapie prion propagation.
5. The arginine-to-glutamine amino acid substitution at codon 211 ($R/Q_{211}$) leaves the mutated $PrP^C$ unable to replicate any classical scrapie agent but has any substantial effect in the susceptibility to the infection with atypical scrapie.
6. The low levels of $PrP^C$ expression in $H_{154}$-Tg563 mice don´t allow us to conclude about the effect of the arginine-to-histidine amino acid exchange at codon 154 ($R/H_{154}$) of the goat $PrP^C$ in susceptibility/resistance to prion infection. However, the positive transmission of some scrapie and BSE isolates suggest a low or null effect.

7. The single isoleucine-to-methionine amino acid exchange at codon 142 ($I/M_{142}$) affects the replication with classical and atypical scrapie agents in a strain-dependent manner; but has any substantial effect on BSE replication.
8. The single glutamine-to-arginine amino acid exchange at codon 171 ($Q/R_{171}$) confers resistance to infection with classical scrapie but not with atypical scrapie.
9. The single asparagine-to-lysine amino acid substitution at codon 176 ($N/K_{176}$) triggers a high level of resistance to infection with classical scrapie and BSE; although it seems to increase the level of susceptibility to atypical scrapie.
10. $H_{154}$-, $Q_{211}$- and $K_{222}$-$PrP^C$ variants in the natural host (goat) protect against the oral infection with classical scrapie. This protective effect is overcome by the intracerebral route in all cases except for $K_{222}$-$PrP^C$ variant which is able to largely protect heterozygous $Q/K_{222}$ goats against the intracerebral infection. Thus, the route of inoculation is a determinant factor in the susceptibility of $H_{154}$-, $Q_{211}$- and $K_{222}$-$PrP^C$ polymorphic variants to classical scrapie infection in goats.
11. Goat-BSE agent produces a genotype dependent transmission to goats where $Q_{211}$-$PrP^C$ variant has an effect limited to prolong incubation periods while $K_{222}$ variant plays a determinant role in the susceptibility of goats to this agent.
12. Similarities in both the natural host and transgenic mice results demonstrate the reliability of transgenic mouse models expressing $PrP^C$ polymorphic variants to predict the genotype-dependent susceptibility to prion infection.

# IX. CONCLUSIONES

1. La línea de ratón Wt-Tg501 es un modelo muy valioso para el estudio de la susceptibilidad de las cabras y ovejas a diferentes cepas de tembladera y EEB así como para la identificación de las cepas de tembladera que afectan a estos pequeños rumiantes. Los estudios de transmisión en otros modelos animales como la línea de ratón BoPrP-Tg110 son útiles para entender la diversidad de cepas de prión existentes.
2. Los estudios de transmisión en los ratones Wt-Tg501 y BoPrP-Tg110 muestran la existencia de al menos cuatro cepas de tembladera clásica circulando por Europa con una distribución regional. Por el contrario, la tembladera atípica parece ser una enfermedad homogénea (sólo una cepa) con potencial para la transmisión natural tanto en el contexto de PrP caprina como bovina.
3. Los ratones de cabra Wt-Tg501 muestran una baja o nula barrera de transmisión a la EEB clásica de vaca pero moderada o alta barrera a la EEB atípica L de vaca, lo que resalta el efecto de la cepa en la barrera de especie bovina-cabra.
4. Los estudios de transmisión en ratones transgénicos que expresan diferentes variantes polimórficas de la $PrP^C$ nos permitieron concluir que el único cambio aminoacídico glutamina-lisina en el codón 222 ($Q/K_{222}$) produce resistencia a la infección con los agentes de tembladera clásica, EEB clásica de vaca y EEB-L atípica de vaca pero no a los agentes de tembladera atípica, EEB de oveja o EEB de cabra. Es más, la variante de la $PrP^C$ $K_{222}$ proporciona un efecto dominante negativo sobre la secuencia de $PrP^C$ *wt* que resulta en una reducción de la eficacia de propagación de la tembladera clásica.
5. El cambio aminoacídico arginina-glutamina en el codón 211 ($R/Q_{211}$) conlleva la inutilidad de la $PrP^C$ mutada de replicar cualquier agente de tembladera clásica aunque no tiene ningún efecto sustancial en la susceptibilidad a la infección con el agente de tembladera atípica.
6. Los bajos niveles de expresión de la $PrP^C$ en los ratones $H_{154}$-Tg563 no nos permiten concluir sobre el efecto del cambio aminoacídico arginina-

histidina en el codón 154 ($R/H_{154}$) en la susceptibilidad/resistencia a la infección con priones. Sin embargo, la transmisión positiva de algunos aislados de tembladera y EEB en estos ratones sugiere un bajo o nulo efecto del cambio aminoacídico $R/H_{154}$.

7. El cambio aminoacídico isoleucina-metionina en el codón 142 ($I/M_{142}$) tiene un efecto en la replicación con agentes de tembladera clásica y atípica que depende de la cepa; pero no tiene ningún efecto sustancial en la replicación de la EEB.
8. El cambio aminoacídico glutamina-arginina en el codón 171 ($Q/R_{171}$) confiere resistencia a la infección con el agente de tembladera clásica pero no de tembladera atípica.
9. El cambio aminoacídico asparragina-lisina en el codón 176 ($N/K_{176}$) confiere alta resistencia a la infección con los agentes de tembladera clásica y EEB; aunque parece aumentar la susceptibilidad a la tembladera atípica.
10. Las variantes polimórficas de la $PrP^C$ $H_{154}$-, $Q_{211}$- y $K_{222}$- protegen al hospedador natural (cabras) frente a la infección oral por tembladera clásica. Este efecto protector es revocado por vía intracerebral en todos los casos excepto en el de la variante de la $PrP^C$ $K_{222}$ que protege casi totalmente a las cabras heterozigotas $Q/K_{222}$ frente a la infección intracerebral por tembladera clásica. Por lo tanto, la ruta de inoculación es un factor determinante en la susceptibilidad de las variantes polimórficas $H_{154}$-, $Q_{211}$- y $K_{222}$- a la infección con este agente de prión.
11. La transmisión del agente EEB de cabra a las cabras es dependiente de genotipo: la variante polimórfica $Q_{211}$ tiene un efecto limitado a prolongar los tiempos de incubación mientras que la variante $K_{222}$ juega un papel fundamental en la susceptibilidad de las cabras a la EEB de cabra.
12. Las similitudes en los resultados tanto en el huésped natural como en los ratones transgénicos demuestran la idoneidad de los modelos de ratón transgénico que expresan diferentes variantes de la $PrP^C$ para predecir la susceptibilidad a la infección con priones en función del genotipo de la PrP.

# X. BIBLIOGRAPHY

(BIOHAZ), E. F. S. A. o. B. H. 2011. Joint scientific opinion on any possible epidemiological or molecular association between tses in animals and humans. . EFSA Journal. 9:1945–2056 [cited 11 Sep 2013].

Abid, K., R. Morales, and C. Soto. 2010. Cellular factors implicated in prion replication. FEBS Lett 584: 2409-2414.

Acin, C. et al. 2013. Prion protein gene variability in spanish goats. Inference through susceptibility to classical scrapie strains and pathogenic distribution of peripheral prp(sc.). PLoS One 8: e61118.

Acutis, P. L. et al. 2006. Identification of prion protein gene polymorphisms in goats from italian scrapie outbreaks. J Gen Virol 87: 1029-1033.

Adler, V. et al. 2003. Small, highly structured rnas participate in the conversion of human recombinant prp(sen) to prp(res) in vitro. J Mol Biol 332: 47-57.

Agrimi, U. et al. 2003. Animal transmissible spongiform encephalopathies and genetics. Vet Res Commun 27 Suppl 1: 31-38.

Aguilar-Calvo, P. et al. 2014. Role of the goat k222-prpc polymorphic variant in prion infection resistance. J Virol 88: 2670-2676.

Aguzzi, A., M. Heikenwalder, and M. Polymenidou. 2007. Insights into prion strains and neurotoxicity. Nat Rev Mol Cell Biol 8: 552-561.

Aguzzi, A., and C. J. Sigurdson. 2004. Antiprion immunotherapy: To suppress or to stimulate? Nat Rev Immunol 4: 725-736.

Almberg, E. S., P. C. Cross, C. J. Johnson, D. M. Heisey, and B. J. Richards. 2011. Modeling routes of chronic wasting disease transmission: Environmental prion persistence promotes deer population decline and extinction. PLoS One 6: e19896.

Alper, T., W. A. Cramp, D. A. Haig, and M. C. Clarke. 1967. Does the agent of scrapie replicate without nucleic acid? Nature 214: 764-766.

Alperovitch, A. et al. 1999. Codon 129 prion protein genotype and sporadic creutzfeldt-jakob disease. Lancet 353: 1673-1674.

Alpers, M. P. 2008. Review. The epidemiology of kuru: Monitoring the epidemic from its peak to its end. Philos Trans R Soc Lond B Biol Sci 363: 3707-3713.

Andreoletti, O. et al. 2000. Early accumulation of prp(sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a romanov flock with natural scrapie. J Gen Virol 81: 3115-3126.

Andreoletti, O. et al. 2002. Prp(sc) accumulation in placentas of ewes exposed to natural scrapie: Influence of foetal prp genotype and effect on ewe-to-lamb transmission. J Gen Virol 83: 2607-2616.

Andreoletti, O. et al. 2006. Bovine spongiform encephalopathy agent in spleen from an arr/arr orally exposed sheep. J Gen Virol 87: 1043-1046.

Andreoletti, O. et al. 2011. Atypical/nor98 scrapie infectivity in sheep peripheral tissues. PLoS Pathog 7: e1001285.

Angers, R. C. et al. 2010. Prion strain mutation determined by prion protein conformational compatibility and primary structure. Science 328: 1154-1158.

Apetri, A. C., K. Surewicz, and W. K. Surewicz. 2004. The effect of disease-associated mutations on the folding pathway of human prion protein. J Biol Chem 279: 18008-18014.

Arsac, J. N. et al. 2007. Similar biochemical signatures and prion protein genotypes in atypical scrapie and nor98 cases, france and norway. Emerg Infect Dis 13: 58-65.

Ballerini, C. et al. 2006. Functional implication of cellular prion protein in antigen-driven interactions between t cells and dendritic cells. J Immunol 176: 7254-7262.

Bannach, O. et al. 2012. Detection of prion protein particles in blood plasma of scrapie infected sheep. PLoS One 7: e36620.

Barillet, F. et al. 2009. Identification of seven haplotypes of the caprine prp gene at codons 127, 142, 154, 211, 222 and 240 in french alpine and saanen breeds and their association with classical scrapie. J Gen Virol 90: 769-776.

Baron, T., A. Bencsik, A. G. Biacabe, E. Morignat, and R. A. Bessen. 2007. Phenotypic similarity of transmissible mink encephalopathy in cattle and l-type bovine spongiform encephalopathy in a mouse model. Emerg Infect Dis 13: 1887-1894.

Baron, T. et al. 2011. Emergence of classical bse strain properties during serial passages of h-bse in wild-type mice. PLoS One 6: e15839.

Barria, M. A., G. C. Telling, P. Gambetti, J. A. Mastrianni, and C. Soto. 2011. Generation of a new form of human prp(sc) in vitro by interspecies transmission from cervid prions. J Biol Chem 286: 7490-7495.

Barron, R. M. et al. 2001. Changing a single amino acid in the n-terminus of murine prp alters tse incubation time across three species barriers. Embo J 20: 5070-5078.

Bartz, J. C., C. Dejoia, T. Tucker, A. E. Kincaid, and R. A. Bessen. 2005. Extraneural prion neuroinvasion without lymphoreticular system infection. J Virol 79: 11858-11863.

Bartz, J. C., D. I. McKenzie, R. A. Bessen, R. F. Marsh, and J. M. Aiken. 1994. Transmissible mink encephalopathy species barrier effect between ferret and mink: Prp gene and protein analysis. J Gen Virol 75: 2947-2953.

Basler, K. et al. 1986. Scrapie and cellular prp isoforms are encoded by the same chromosomal gene. Cell 46: 417-428.

Baylis, M. et al. 2004. Risk of scrapie in british sheep of different prion protein genotype. J Gen Virol 85: 2735-2740.

Beck, J., J. Collinge, and S. Mead. 2012. Prion protein gene m232r variation is probably an uncommon polymorphism rather than a pathogenic mutation. Brain 135: e209.

Behrens, A. et al. 2002. Absence of the prion protein homologue doppel causes male sterility. Embo J 21: 3652-3658.

Belay, E. D. 1999. Transmissible spongiform encephalopathies in humans. Annu Rev Microbiol 53: 283-314.

Belt, P. B. et al. 1995. Identification of five allelic variants of the sheep prp gene and their association with natural scrapie. J Gen Virol 76: 509-517.

Bellworthy, S. J. et al. 2008. Oral transmission of bse to vrq/vrq sheep in an experimental flock. Vet Rec 162: 130-131.

Bellworthy, S. J. et al. 2005. Tissue distribution of bovine spongiform encephalopathy infectivity in romney sheep up to the onset of clinical disease after oral challenge. Vet Rec 156: 197-202.

Bencsik, A., and T. Baron. 2007. Bovine spongiform encephalopathy agent in a prion protein (prp)arr/arr genotype sheep after peripheral challenge: Complete immunohistochemical analysis of disease-associated prp and transmission studies to ovine-transgenic mice. J Infect Dis 195: 989-996.

Benestad, S. L., J. N. Arsac, W. Goldmann, and M. Noremark. 2008. Atypical/nor98 scrapie: Properties of the agent, genetics, and epidemiology. Vet Res 39: 19.

Benestad, S. L., L. Austbo, M. A. Tranulis, A. Espenes, and I. Olsaker. 2012. Healthy goats naturally devoid of prion protein. Vet Res 43: 87.

Benestad, S. L. et al. 2003. Cases of scrapie with unusual features in norway and designation of a new type, nor98. Vet Rec 153: 202-208.

Beringue, V. et al. 2007. A bovine prion acquires an epidemic bovine spongiform encephalopathy strain-like phenotype on interspecies transmission. J Neurosci 27: 6965-6971.

Beringue, V. et al. 2006. Isolation from cattle of a prion strain distinct from that causing bovine spongiform encephalopathy. PLoS Pathog 2.

Beringue, V. et al. 2008a. Transmission of atypical bovine prions to mice transgenic for human prion protein. Emerg Infect Dis 14: 1898-1901.

Beringue, V., J. L. Vilotte, and H. Laude. 2008b. Prion agent diversity and species barrier. Vet Res 39: 47.

Beringue, V., J. L. Vilotte, and H. Laude. 2008c. Prion agents diversity and species barrier. Vet Res 39: 47.

Bessen, R. A., and R. F. Marsh. 1992. Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. J Virol 66: 2096-2101.

Bett, C. et al. 2012a. Structure of the beta2-alpha2 loop and interspecies prion transmission. FASEB J 26: 2868-2876.

Bett, C. et al. 2012b. Biochemical properties of highly neuroinvasive prion strains. PLoS Pathog 8: e1002522.

Biacabe, A. G., J. L. Laplanche, S. Ryder, and T. Baron. 2004. Distinct molecular phenotypes in bovine prion diseases. EMBO Rep 5: 110-115.

Biljan, I. et al. 2012. Structural basis for the protective effect of the human prion protein carrying the dominant-negative e219k polymorphism. Biochem J 446: 243-251.

Billinis, C. et al. 2002. Prion protein gene polymorphisms in natural goat scrapie. J Gen Virol 83: 713-721.

Bons, N., S. Lehmann, N. Mestre-Frances, D. Dormont, and P. Brown. 2002. Brain and buffy coat transmission of bovine spongiform encephalopathy to the primate microcebus murinus. Transfusion 42: 513-516.

Borchelt, D. R. et al. 1996. A vector for expressing foreign genes in the brains and hearts of transgenic mice. Genet Anal 13: 159-163.

Borchelt, D. R., M. Scott, A. Taraboulos, N. Stahl, and S. B. Prusiner. 1990. Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. J Cell Biol 110: 743-752.

Borchelt, D. R., A. Taraboulos, and S. B. Prusiner. 1992. Evidence for synthesis of scrapie prion proteins in the endocytic pathway. J Biol Chem 267: 16188-16199.

Bossers, A. et al. 1997. Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms. Proc Natl Acad Sci U S A 94: 4931-4936.

Bossers, A., R. de Vries, and M. A. Smits. 2000. Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of prp. J Virol 74: 1407-1414.

Bossers, A., B. E. Schreuder, I. H. Muileman, P. B. Belt, and M. A. Smits. 1996. Prp genotype contributes to determining survival times of sheep with natural scrapie. J Gen Virol 77: 2669-2673.

Bouzalas, I. G. et al. 2010. Caprine prnp polymorphisms at codons 171, 211, 222 and 240 in a greek herd and their association with classical scrapie. J Gen Virol 91: 1629-1634.

Bradley, R., and J. W. Wilesmith. 1993. Epidemiology and control of bovine spongiform encephalopathy (bse). Br Med Bull 49: 932-959.

Brandner, S. et al. 1996. Normal host prion protein necessary for scrapie-induced neurotoxicity. Nature 379: 339-343.

Brown, D. A., M. E. Bruce, and J. R. Fraser. 2003. Comparison of the neuropathological characteristics of bovine spongiform encephalopathy (bse) and variant creutzfeldt-jakob disease (vcjd) in mice. Neuropathol Appl Neurobiol 29: 262-272.

Brown, D. R., C. Clive, and S. J. Haswell. 2001. Antioxidant activity related to copper binding of native prion protein. J Neurochem 76: 69-76.

Brown, H. R. et al. 1990. The mrna encoding the scrapie agent protein is present in a variety of non-neuronal cells. Acta Neuropathol 80: 1-6.

Brown, P., M. A. Preece, and R. G. Will. 1992. "Friendly fire" In medicine: Hormones, homografts, and creutzfeldt- jakob disease. Lancet 340: 24-27.

Brown, P. et al. 1998. The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. Transfusion 38: 810-816.

Bruce, M. E., and A. G. Dickinson. 1987. Biological evidence that scrapie agent has an independent genome. J Gen Virol 68: 79-89.

Bruce, M. E. et al. 1997. Transmissions to mice indicate that 'new variant' cjd is caused by the bse agent. Nature 389: 498-501.

Bucalossi, C. et al. 2011. Assessment of the genetic susceptibility of sheep to scrapie by protein misfolding cyclic amplification and comparison with experimental scrapie transmission studies. J Virol 85: 8386-8392.

Bueler, H. et al. 1993. Mice devoid of prp are resistant to scrapie. Cell 73: 1339-1347.

Buschmann, A. et al. 2006. Atypical bse in germany--proof of transmissibility and biochemical characterization. Vet Microbiol 117: 103-116.

Buschmann, A., and M. H. Groschup. 2005. Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle. J Infect Dis 192: 934-942.

Campbell, L. et al. 2013. The prp(c) c1 fragment derived from the ovine a136r154r171prnp allele is highly abundant in sheep brain and inhibits fibrillisation of full-length prp(c) protein in vitro. Biochim Biophys Acta 1832: 826-836.

Capobianco, R. et al. 2007. Conversion of the base prion strain into the bse strain: The origin of bse? PLoS Pathog 3: e31.

Casalone, C. et al. 2004. Identification of a second bovine amyloidotic spongiform encephalopathy: Molecular similarities with sporadic creutzfeldt-jakob disease. Proc Natl Acad Sci U S A 101: 3065-3070.

Castilla, J. et al. 2005a. Vertical transmission of bovine spongiform encephalopathy prions evaluated in a transgenic mouse model. J Virol 79: 8665-8668.

Castilla, J. et al. 2003. Early detection of prp(res) in bse-infected bovine prp transgenic mice. Arch Virol 148: 677-691.

Castilla, J., P. Saa, C. Hetz, and C. Soto. 2005b. In vitro generation of infectious scrapie prions. Cell 121: 195-206.

Caughey, B., G. S. Baron, B. Chesebro, and M. Jeffrey. 2009. Getting a grip on prions: Oligomers, amyloids, and pathological membrane interactions. Annu Rev Biochem 78: 177-204.

Caughey, B., and B. Chesebro. 2001. Transmissible spongiform encephalopathies and prion protein interconversions. Adv Virus Res 56: 277-311.

Caughey, B., and P. T. Lansbury. 2003. Protofibrils, pores, fibrils, and neurodegeneration: Separating the responsible protein aggregates from the innocent bystanders. Annu Rev Neurosci 26: 267-298.

Caughey, B., and G. J. Raymond. 1991. The scrapie-associated form of prp is made from a cell surface precursor that is both protease- and phospholipase-sensitive. J Biol Chem 266: 18217-18223.

Cobb, N. J., and W. K. Surewicz. 2009. Prion diseases and their biochemical mechanisms. Biochemistry 48: 2574-2585.

Colby, D. W. et al. 2009. Design and construction of diverse mammalian prion strains. Proc Natl Acad Sci U S A 106: 20417-20422.

Colby, D. W., and S. B. Prusiner. 2011. Prions. Cold Spring Harb Perspect Biol 3: a006833.

Colby, D. W. et al. 2010. Protease-sensitive synthetic prions. PLoS Pathog 6: e1000736.

Colchester, A. C., and N. T. Colchester. 2005. The origin of bovine spongiform encephalopathy: The human prion disease hypothesis. Lancet 366: 856-861.

Colussi, S. et al. 2008. Histidine at codon 154 of the prion protein gene is a risk factor for nor98 scrapie in goats. J Gen Virol 89: 3173-3176.

Collinge, J. 2001. Prion diseases of humans and animals: Their causes and molecular basis. Annu Rev Neurosci 24: 519-550.

Collinge, J., and A. R. Clarke. 2007. A general model of prion strains and their pathogenicity. Science 318: 930-936.

Collinge, J., and M. S. Palmer. 1994. Molecular genetics of human prion diseases. Philos Trans R Soc Lond B Biol Sci 343: 371-378.

Collinge, J., M. S. Palmer, and A. J. Dryden. 1991. Genetic predisposition to iatrogenic creutzfeldt-jakob disease. Lancet 337: 1441-1442.

Collinge, J., K. C. Sidle, J. Meads, J. Ironside, and A. F. Hill. 1996. Molecular analysis of prion strain variation and the aetiology of 'new variant' cjd. Nature 383: 685-690.

Collinge, J. et al. 1994. Prion protein is necessary for normal synaptic function. Nature 370: 295-297.

Comoy, E. E. et al. 2008. Atypical bse (base) transmitted from asymptomatic aging cattle to a primate. PLoS One 3: e3017.

Corbiere, F. et al. 2013a. The limits of test-based scrapie eradication programs in goats. PLoS One 8: e54911.

Corbiere, F. et al. 2013b. Prp-associated resistance to scrapie in five highly infected goat herds. J Gen Virol 94: 241-245.

Cordeiro, Y. et al. 2001. DNA converts cellular prion protein into the beta-sheet conformation and inhibits prion peptide aggregation. J Biol Chem 276: 49400-49409.

Cotto, E., M. Andre, J. Forgue, H. J. Fleury, and P. J. Babin. 2005. Molecular characterization, phylogenetic relationships, and developmental expression patterns of prion genes in zebrafish (danio rerio). Febs J 272: 500-513.

Crozet, C. et al. 2004. Inhibition of prpsc formation by lentiviral gene transfer of prp containing dominant negative mutations. J Cell Sci 117: 5591-5597.

Chen, S., A. Mange, L. Dong, S. Lehmann, and M. Schachner. 2003. Prion protein as trans-interacting partner for neurons is involved in neurite outgrowth and neuronal survival. Mol Cell Neurosci 22: 227-233.

Chen, S. G. et al. 1995. Truncated forms of the human prion protein in normal brain and in prion diseases. J Biol Chem 270: 19173-19180.

Chesebro, B. 1998. Bse and prions: Uncertainties about the agent. Science 279: 42-43.

Chiarini, L. B. et al. 2002. Cellular prion protein transduces neuroprotective signals. Embo J 21: 3317-3326.

Dassanayake, R. P. et al. 2012. Cell-surface expression of prpc and the presence of scrapie prions in the blood of goats. J Gen Virol 93: 1127-1131.

Dassanayake, R. P. et al. 2011. Classical scrapie prions in ovine blood are associated with b lymphocytes and platelet-rich plasma. BMC Vet Res 7: 75.

Dawson, M., L. J. Hoinville, B. D. Hosie, and N. Hunter. 1998. Guidance on the use of prp genotyping as an aid to the control of clinical scrapie. Scrapie information group. Vet Rec 142: 623-625.

de Almeida, C. J. et al. 2005. The cellular prion protein modulates phagocytosis and inflammatory response. J Leukoc Biol 77: 238-246.

DebBurman, S. K., G. J. Raymond, B. Caughey, and S. Lindquist. 1997. Chaperone-supervised conversion of prion protein to its protease- resistant form. Proc Natl Acad Sci U S A 94: 13938-13943.

Deleault, N. R. et al. 2005. Prpres amplification reconstituted with purified prion proteins and synthetic polyanions. J Biol Chem.

Deleault, N. R., R. W. Lucassen, and S. Supattapone. 2003. Rna molecules stimulate prion protein conversion. Nature 425: 717-720.

Deleault, N. R. et al. 2012. Cofactor molecules maintain infectious conformation and restrict strain properties in purified prions. Proc Natl Acad Sci U S A 109: E1938-1946.

Dickinson, A. G. 1976. Scrapie in sheep and goats. Front Biol 44: 209-241.

Doh-ura, K., T. Kitamoto, Y. Sakaki, and J. Tateishi. 1991. Cjd discrepancy. Nature 353: 801-802.

Eghiaian, F. et al. 2004. Insight into the prpc-->prpsc conversion from the structures of antibody-bound ovine prion scrapie-susceptibility variants. Proc Natl Acad Sci U S A 101: 10254-10259.

Eiden, M., E. O. Soto, T. C. Mettenleiter, and M. H. Groschup. 2011. Effects of polymorphisms in ovine and caprine prion protein alleles on cell-free conversion. Vet Res 42: 30.

Eloit, M. et al. 2005. Bse agent signatures in a goat. Vet Rec 156: 523-524.

Espinosa, J. C. et al. 2007a. Sheep-passaged bovine spongiform encephalopathy agent exhibits altered pathobiological properties in bovine-prp transgenic mice. J Virol 81: 835-843.

Espinosa, J. C. et al. 2009. Transgenic mice expressing porcine prion protein resistant to classical scrapie but susceptible to sheep bovine spongiform encephalopathy and atypical scrapie. Emerg Infect Dis 15: 1214-1221.

Espinosa, J. C., M. Morales, J. Castilla, M. Rogers, and J. M. Torres. 2007b. Progression of prion infectivity in asymptomatic cattle after oral bovine spongiform encephalopathy challenge. J Gen Virol 88: 1379-1383.

European, P., and Council. 2005. Commission regulation (ec) no 36/2005 of 12 january 2005 amending annexes iii and x to regulation (ec) no 999/2001 of the european parliament and of the council as regards epidemio-surveillance for transmissible spongiform encephalopathies in bovine, ovine and caprine animals. Text with eea relevance.

Falsig, J., K. P. Nilsson, T. P. Knowles, and A. Aguzzi. 2008. Chemical and biophysical insights into the propagation of prion strains. HFSP J 2: 332-341.

Fediaevsky, A., P. Gasqui, D. Calavas, and C. Ducrot. 2010. Discrepant epidemiological patterns between classical and atypical scrapie in sheep flocks under french tse control measures. Vet J 185: 338-340.

Feraudet, C. et al. 2005. Screening of 145 anti-prp monoclonal antibodies for their capacity to inhibit prpsc replication in infected cells. J. Biol. Chem. 280: 11247-11258.

Fitzmaurice, T. J. et al. 2008. The stability and aggregation of ovine prion protein associated with classical and atypical scrapie correlates with the ease of unwinding of helix-2. Biochem J 409: 367-375.

Foster, J. D., M. Bruce, I. McConnell, A. Chree, and H. Fraser. 1996a. Detection of bse infectivity in brain and spleen of experimentally infected sheep. Vet Rec 138: 546-548.

Foster, J. D., J. Hope, and H. Fraser. 1993. Transmission of bovine spongiform encephalopathy to sheep and goats. Vet Rec 133: 339-341.

Foster, J. D., D. Parnham, A. Chong, W. Goldmann, and N. Hunter. 2001a. Clinical signs, histopathology and genetics of experimental transmission of bse and natural scrapie to sheep and goats. Vet Rec 148: 165-171.

Foster, J. D., D. W. Parnham, N. Hunter, and M. Bruce. 2001b. Distribution of the prion protein in sheep terminally affected with bse following experimental oral transmission. J Gen Virol 82: 2319-2326.

Foster, J. D., M. Wilson, and N. Hunter. 1996b. Immunolocalisation of the prion protein (prp) in the brains of sheep with scrapie. Vet Rec 139: 512-515.

Fragkiadaki, E. G. et al. 2011. Prnp genetic variability and molecular typing of natural goat scrapie isolates in a high number of infected flocks. Vet Res 42: 104.

Fraser, H., and A. G. Dickinson. 1968. The sequential development of the brain lesion of scrapie in three strains of mice. J Comp Pathol 78: 301-311.

Gabriel, J. M., B. Oesch, H. Kretzschmar, M. Scott, and S. B. Prusiner. 1992. Molecular cloning of a candidate chicken prion protein. Proc Natl Acad Sci U S A 89: 9097-9101.

Gajdusek, D. C., and C. J. Gibbs, Jr. 1968. Slow, latent and temperate virus infections of the central nervous system. Res Publ Assoc Res Nerv Ment Dis 44: 254-280.

Gajdusek, D. C., C. J. Gibbs, Jr., and M. Alpers. 1967. Transmission and passage of experimenal "Kuru" To chimpanzees. Science 155: 212-214.

Gajdusek, D. C., and V. Zigas. 1957. Degenerative disease of the central nervous system in new guinea; the endemic occurrence of kuru in the native population. N Engl J Med 257: 974-978.

Gambetti, P., Q. Kong, W. Zou, P. Parchi, and S. G. Chen. 2003. Sporadic and familial cjd: Classification and characterisation. Br Med Bull 66: 213-239.

Gambetti, P., P. Parchi, R. B. Petersen, S. G. Chen, and E. Lugaresi. 1995. Fatal familial insomnia and familial creutzfeldt-jakob disease: Clinical, pathological and molecular features. Brain Pathol 5: 43-51.

Garza, M. C., M. Monzon, B. Marin, J. J. Badiola, and E. Monleon. 2014. Distribution of peripheral prpsc in sheep with naturally acquired scrapie. PLoS One 9: e97768.

Geoghegan, J. C., M. B. Miller, A. H. Kwak, B. T. Harris, and S. Supattapone. 2009. Trans-dominant inhibition of prion propagation in vitro is not mediated by an accessory cofactor. PLoS Pathog 5: e1000535.

Gielbert, A. et al. 2009. High-resolution differentiation of transmissible spongiform encephalopathy strains by quantitative n-terminal amino acid profiling (n-taap) of pk-digested abnormal prion protein. J Mass Spectrom 44: 384-396.

Giles, K. et al. 2010. Human prion strain selection in transgenic mice. Ann Neurol 68: 151-161.

Glatzel, M., and A. Aguzzi. 2001. The shifting biology of prions. Brain Res Brain Res Rev 36: 241-248.

Glatzel, M., K. Stoeck, H. Seeger, T. Luhrs, and A. Aguzzi. 2005. Human prion diseases: Molecular and clinical aspects. Arch Neurol 62: 545-552.

Goldmann, W., A. Chong, J. Foster, J. Hope, and N. Hunter. 1998. The shortest known prion protein gene allele occurs in goats, has only three octapeptide repeats and is non-pathogenic. J Gen Virol 79: 3173-3176.

Goldmann, W. et al. 1996. Novel polymorphisms in the caprine prp gene: A codon 142 mutation associated with scrapie incubation period. J Gen Virol 77: 2885-2891.

Goldmann, W. et al. 2011. Caprine prion gene polymorphisms are associated with decreased incidence of classical scrapie in goat herds in the united kingdom. Vet Res 42: 110.

Gonzalez, L. et al. 2007. Comparative titration of experimental ovine bse infectivity in sheep and mice. J Gen Virol 88: 714-717.

Gonzalez, L. et al. 2010. Pathogenesis of natural goat scrapie: Modulation by host prnp genotype and effect of co-existent conditions. Vet Res 41: 48.

Gonzalez, L. et al. 2005. Phenotype of disease-associated prp accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep. J Gen Virol 86: 827-838.

Gotte, D. R. et al. 2011. Atypical scrapie isolates involve a uniform prion species with a complex molecular signature. PLoS One 6: e27510.

Gough, K. C. et al. 2011. The oral secretion of infectious scrapie prions occurs in preclinical sheep with a range of prnp genotypes. J Virol 86: 566-571.

Greenlee, J. J., R. A. Kunkle, J. A. Richt, E. M. Nicholson, and A. N. Hamir. 2014. Lack of prion accumulation in lymphoid tissues of prnp arq/arr sheep intracranially inoculated with the agent of scrapie. PLoS One 9: e108029.

Griffith, J. S. 1967. Self-replication and scrapie. Nature 215: 1043-1044.

Griffiths, P. C. et al. 2010. Characterization of atypical scrapie cases from great britain in transgenic ovine prp mice. J Gen Virol 91: 2132-2138.

Groschup, M. H. et al. 2007. Classic scrapie in sheep with the arr/arr prion genotype in germany and france. Emerg Infect Dis 13: 1201-1207.

Haass, C., and D. J. Selkoe. 2007. Soluble protein oligomers in neurodegeneration: Lessons from the alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol 8: 101-112.

Hadlow, W. J., R. C. Kennedy, and R. E. Race. 1982. Natural infection of suffolk sheep with scrapie virus. J Infect Dis 146: 657-664.

Hagenaars, T. J. et al. 2010. Scrapie prevalence in sheep of susceptible genotype is declining in a population subject to breeding for resistance. BMC Vet Res 6: 25.

Hamir, A. N. et al. 2004. Transmission of sheep scrapie to elk (cervus elaphus nelsoni) by intracerebral inoculation: Final outcome of the experiment. J Vet Diagn Invest 16: 316-321.

Hewitt, P. E., C. A. Llewelyn, J. Mackenzie, and R. G. Will. 2006. Creutzfeldt-jakob disease and blood transfusion: Results of the uk transfusion medicine epidemiological review study. Vox Sang 91: 221-230.

Hill, A. F., and J. Collinge. 2003. Subclinical prion infection in humans and animals. Br Med Bull 66: 161-170.

Hill, A. F. et al. 1997. The same prion strain causes vcjd and bse. Nature 389: 448-450, 526.

Hill, A. F. et al. 1998. Molecular screening of sheep for bovine spongiform encephalopathy. Neurosci Lett 255: 159-162.

Hizume, M. et al. 2009. Human prion protein (prp) 219k is converted to prpsc but shows heterozygous inhibition in variant creutzfeldt-jakob disease infection. J Biol Chem 284: 3603-3609.

Hoffmann, C. et al. 2011. Bse infectivity in jejunum, ileum and ileocaecal junction of incubating cattle. Vet Res 42: 21.

Hoffmann, C. et al. 2007. Prions spread via the autonomic nervous system from the gut to the central nervous system in cattle incubating bovine spongiform encephalopathy. J Gen Virol 88: 1048-1055.

Holznagel, E. et al. 2013. Foodborne transmission of bovine spongiform encephalopathy to nonhuman primates. Emerg Infect Dis 19: 712-720.

Hopp, P., M. K. Omer, and B. T. Heier. 2006. A case-control study of scrapie nor98 in norwegian sheep flocks. J Gen Virol 87: 3729-3736.

Horiuchi, M., S. A. Priola, J. Chabry, and B. Caughey. 2000. Interactions between heterologous forms of prion protein: Binding, inhibition of conversion, and species barriers. Proc Natl Acad Sci U S A 97: 5836-5841.

Horonchik, L. et al. 2005. Heparan sulfate is a cellular receptor for purified infectious prions. J Biol Chem.

Houston, F., J. D. Foster, A. Chong, N. Hunter, and C. J. Bostock. 2000. Transmission of bse by blood transfusion in sheep. Lancet 356: 999-1000.

Hunter, N. 1996. Prion protein (prnp) genotypes and natural scrapie in
closed flocks of cheviot and suffolk sheep in britain. In: D. B. Court L (ed.) Transmissible subacute spongiform encephalopathies:
Prion diseases. p 47–50. Elsevier, Paris.

Hunter, N. 1997. Prp genetics in sheep and the applications for scrapie and bse. Trends Microbiol 5: 331-334.

Hunter, N. 2003. Scrapie and experimental bse in sheep. Br Med Bull 66: 171-183.

Ikeda, T. et al. 1995. Amino acid polymorphisms of prp with reference to onset of scrapie in suffolk and corriedale sheep in japan. J Gen Virol 76: 2577-2581.

Immel, F. et al. 2007. In vitro analysis of spure2p, a prion related protein, exemplifies the relationship between amyloid and prion. J Biol Chem.

Imran, M., and S. Mahmood. 2011. An overview of animal prion diseases. Virol J 8: 493.

Inoue, S., M. Tanaka, M. Horiuchi, N. Ishiguro, and M. Shinagawa. 1997. Characterization of the bovine prion protein gene: The expression requires interaction between the promoter and intron. J Vet Med Sci 59: 175-183.

Iwata, N. et al. 2006. Distribution of prp(sc) in cattle with bovine spongiform encephalopathy slaughtered at abattoirs in japan. Jpn J Infect Dis 59: 100-107.

Jackman, R. et al. 2006. Evaluation of a preclinical blood test for scrapie in sheep using immunocapillary electrophoresis. J AOAC Int 89: 720-727.

Jahandideh, S., M. Jamalan, and M. Faridounnia. 2014. Molecular dynamics study of the dominant-negative e219k polymorphism in human prion protein. J Biomol Struct Dyn: 1-11.

Jeffrey, M., and L. Gonzalez. 2004. Pathology and pathogenesis of bovine spongiform encephalopathy and scrapie. Curr Top Microbiol Immunol 284: 65-97.

Jeffrey, M., and L. Gonzalez. 2007. Classical sheep transmissible spongiform encephalopathies: Pathogenesis, pathological phenotypes and clinical disease. Neuropathol Appl Neurobiol 33: 373-394.

Jeffrey, M. et al. 2014. Incidence of infection in prnp arr/arr sheep following experimental inoculation with or natural exposure to classical scrapie. PLoS One 9: e91026.

Jeffrey, M. et al. 2006. Immunohistochemical features of prp(d) accumulation in natural and experimental goat transmissible spongiform encephalopathies. J Comp Pathol 134: 171-181.

Jouvin-Marche, E. et al. 2006. Overexpression of cellular prion protein induces an antioxidant environment altering t cell development in the thymus. J Immunol 176: 3490-3497.

Kaatz, M. et al. 2012. Spread of classic bse prions from the gut via the peripheral nervous system to the brain. Am J Pathol 181: 515-524.

Kanata, E. et al. 2014. Perspectives of a scrapie resistance breeding scheme targeting q211, s146 and k222 caprine prnp alleles in greek goats. Vet Res 45: 43.

Kaneko, K. et al. 1997. Evidence for protein x binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. Proc Natl Acad Sci U S A 94: 10069-10074.

Kascsak, R. J. et al. 1985. Biochemical differences among scrapie-associated fibrils support the biological diversity of scrapie agents. J Gen Virol 66: 1715-1722.

Kaski, d. et al. 2009. Variant cjd in an individual heterozygous for prnp codon 129. Lancet 374: 2128.

Kim, J. I. et al. 2010. Mammalian prions generated from bacterially expressed prion protein in the absence of any mammalian cofactors. J Biol Chem.

Kimberlin, R. H. 1982. Scrapie agent: Prions or virinos? Nature 297: 107-108.

Kimberlin, R. H., S. Cole, and C. A. Walker. 1987. Temporary and permanent modifications to a single strain of mouse scrapie on transmission to rats and hamsters. J Gen Virol 68: 1875-1881.

Kimberlin, R. H., C. A. Walker, and H. Fraser. 1989. The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. J Gen Virol 70: 2017-2025.

Kittelberger, R. et al. 2010. Atypical scrapie/nor98 in a sheep from new zealand. J Vet Diagn Invest 22: 863-875.

Kobayashi, A., M. Hizume, K. Teruya, S. Mohri, and T. Kitamoto. 2009. Heterozygous inhibition in prion infection: The stone fence model. Prion 3: 27-30.

Konold, T. et al. 2013. Evidence of effective scrapie transmission via colostrum and milk in sheep. BMC Vet Res 9: 99.

Kovacs, G. G., and H. Budka. 2009. Molecular pathology of human prion diseases. Int J Mol Sci 10: 976-999.

Kretzschmar, H. A., S. B. Prusiner, L. E. Stowring, and S. J. DeArmond. 1986. Scrapie prion proteins are synthesized in neurons. Am J Pathol 122: 1-5.

Kuczius, T. et al. 2007. Regional and phenotype heterogeneity of cellular prion proteins in the human brain. Eur J Neurosci.

Kujala, P. et al. 2011. Prion uptake in the gut: Identification of the first uptake and replication sites. PLoS Pathog 7: e1002449.

Kuznetsova, A., D. McKenzie, P. Banser, T. Siddique, and J. M. Aiken. 2014. Potential role of soil properties in the spread of cwd in western canada. Prion 8.

Lacroux, C. et al. 2014a. Genetic resistance to scrapie infection in experimentally challenged goats. J Virol.

Lacroux, C. et al. 2014b. Scrapie prions: An etiologic agent of scjd in human? Prion 8 Supplement: 1.

Lacroux, C. et al. 2012. Prionemia and leukocyte-platelet-associated infectivity in sheep transmissible spongiform encephalopathy models. J Virol 86: 2056-2066.

Laffont-Proust, I. et al. 2005. The n-terminal cleavage of cellular prion protein in the human brain. FEBS Lett.

Lasmezas, C. I. et al. 2005. Risk of oral infection with bovine spongiform encephalopathy agent in primates. Lancet 365: 781-783.

Lasmezas, C. I. et al. 1997. Transmission of the bse agent to mice in the absence of detectable abnormal prion protein. Science 275: 402-405.

Laude, H. et al. 2002. New in vivo and ex vivo models for the experimental study of sheep scrapie: Development and perspectives. C R Biol 325: 49-57.

Lauren, J., D. A. Gimbel, H. B. Nygaard, J. W. Gilbert, and S. M. Strittmatter. 2009. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature 457: 1128-1132.

Le Dur, A. et al. 2005. A newly identified type of scrapie agent can naturally infect sheep with resistant prp genotypes. Proc Natl Acad Sci U S A.

Lee, C. I., Q. Yang, V. Perrier, and I. V. Baskakov. 2007. The dominant-negative effect of the q218k variant of the prion protein does not require protein x. Protein Sci 16: 2166-2173.

Legname, G. et al. 2004. Synthetic mammalian prions. Science 305: 673-676.

Legname, G. et al. 2006. Continuum of prion protein structures enciphers a multitude of prion isolate-specified phenotypes. Proc Natl Acad Sci U S A.

Lezmi, S., T. Seuberlich, A. Oevermann, T. Baron, and A. Bencsik. 2011. Comparison of brain prpd distribution in ovine bse and scrapie. Vet Pathol 48: 1101-1108.

Li, J., S. Browning, S. P. Mahal, A. M. Oelschlegel, and C. Weissmann. 2010. Darwinian evolution of prions in cell culture. Science 327: 869-872.

Liemann, S., and R. Glockshuber. 1999. Influence of amino acid substitutions related to inherited human prion diseases on the thermodynamic stability of the cellular prion protein. Biochemistry 38: 3258-3267.

Ligios, C. et al. 2011. Sheep with scrapie and mastitis transmit infectious prions through the milk. J Virol 85: 1136-1139.

Ligios, C. et al. 2005. Prp(sc) in mammary glands of sheep affected by scrapie and mastitis. Nat Med 11: 1137-1138.

Linden, R. et al. 2008. Physiology of the prion protein. Physiol Rev 88: 673-728.

Luhken, G., A. Buschmann, M. H. Groschup, and G. Erhardt. 2004. Prion protein allele a136 h154q171 is associated with high susceptibility to scrapie in purebred and crossbred german merinoland sheep. Arch Virol 149: 1571-1580.

Llewelyn, C. A. et al. 2004. Possible transmission of variant creutzfeldt-jakob disease by blood transfusion. Lancet 363: 417-421.

Lloyd, S., S. Mead, and J. Collinge. 2011. Genetics of prion disease. Top Curr Chem.

Lloyd, S. E., S. Mead, and J. Collinge. 2013. Genetics of prion diseases. Curr Opin Genet Dev 23: 345-351.

Maestrale, C. et al. 2009. P.Asn176lys and p.Met137thr dimorphisms of the prnp gene significantly decrease the susceptibility to classical scrapie in arq/arq sheep. Anim Genet 40: 982-985.

Mahal, S. P., E. A. Asante, M. Antoniou, and J. Collinge. 2001. Isolation and functional characterisation of the promoter region of the human prion protein gene. Gene 268: 105-114.

Mahal, S. P. et al. 2007. Prion strain discrimination in cell culture: The cell panel assay. Proc Natl Acad Sci U S A 104: 20908-20913.

Makarava, N. et al. 2010. Recombinant prion protein induces a new transmissible prion disease in wild-type animals. Acta Neuropathol 119: 177-187.

Manson, J. C. et al. 1994. 129/ola mice carrying a null mutation in prp that abolishes mrna production are developmentally normal. Mol Neurobiol 8: 121-127.

Manson, J. C. et al. 1999. A single amino acid alteration (101l) introduced into murine prp dramatically alters incubation time of transmissible spongiform encephalopathy. Embo J 18: 6855-6864.

Manuelidis, E. E., J. Kim, J. N. Angelo, and L. Manuelidis. 1976. Serial propagation of creutzfeldt-jakob disease in guinea pigs. Proc Natl Acad Sci U S A 73: 223-227.

Manuelidis, L. 2013. Infectious particles, stress, and induced prion amyloids: A unifying perspective. Virulence 4: 373-383.

Marella, M., S. Lehmann, J. Grassi, and J. Chabry. 2002. Filipin prevents pathological prion protein accumulation by reducing endocytosis and inducing cellular prp release. J Biol Chem 277: 25457-25464.

Marsh, R. F., A. E. Kincaid, R. A. Bessen, and J. C. Bartz. 2005. Interspecies transmission of chronic wasting disease prions to squirrel monkeys (saimiri sciureus). J Virol 79: 13794-13796.

Masters, C. L. et al. 1979. Creutzfeldt-jakob disease: Patterns of worldwide occurrence and the significance of familial and sporadic clustering. Ann Neurol 5: 177-188.

Mathiason, C. K. et al. 2010. B cells and platelets harbor prion infectivity in the blood of cwd-infected deer. J Virol.

Mathiason, C. K. et al. 2006. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science 314: 133-136.

Mattei, V. et al. 2004. Prion protein is a component of the multimolecular signaling complex involved in t cell activation. FEBS Lett 560: 14-18.

McKnight, S., and R. Tjian. 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell 46: 795-805.

McLennan, N. F. et al. 2004. Prion protein accumulation and neuroprotection in hypoxic brain damage. Am J Pathol 165: 227-235.

Mead, S. et al. 2003. Balancing selection at the prion protein gene consistent with prehistoric kurulike epidemics. Science 300: 640-643.

Megy, S. et al. 2004. Possible role of region 152-156 in the structural duality of a peptide fragment from sheep prion protein. Protein Sci 13: 3151-3160.

Mehrpour, M., and P. Codogno. 2010. Prion protein: From physiology to cancer biology. Cancer Lett 290: 1-23.

Miller, M. W., M. A. Wild, and E. S. Williams. 1998. Epidemiology of chronic wasting disease in captive rocky mountain elk. J Wildl Dis 34: 532-538.

Miller, M. W., E. S. Williams, N. T. Hobbs, and L. L. Wolfe. 2004. Environmental sources of prion transmission in mule deer. Emerg Infect Dis 10: 1003-1006.

Mishra, R. S. et al. 2004. Protease-resistant human prion protein and ferritin are cotransported across caco-2 epithelial cells: Implications for species barrier in prion uptake from the intestine. J Neurosci 24: 11280-11290.

Mohan, J., K. L. Brown, C. F. Farquhar, M. E. Bruce, and N. A. Mabbott. 2004. Scrapie transmission following exposure through the skin is dependent on follicular dendritic cells in lymphoid tissues. J Dermatol Sci 35: 101-111.

Moore, R. C. et al. 1999. Ataxia in prion protein (prp)-deficient mice is associated with upregulation of the novel prp-like protein doppel. J Mol Biol 292: 797-817.

Moore, S. J., M. Simmons, M. Chaplin, and J. Spiropoulos. 2008. Neuroanatomical distribution of abnormal prion protein in naturally occurring atypical scrapie cases in great britain. Acta Neuropathol 116: 547-559.

Muras, A. G. et al. 2009. Prion protein ablation increases cellular aggregation and embolization contributing to mechanisms of metastasis. Int J Cancer 125: 1523-1531.

Nalls, A. V. et al. 2013. Mother to offspring transmission of chronic wasting disease in reeves' muntjac deer. PLoS One 8: e71844.

Neutra, M. R., E. Pringault, and J. P. Kraehenbuhl. 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 14: 275-300.

Nicolas, O., R. Gavin, and J. A. del Rio. 2009. New insights into cellular prion protein (prpc) functions: The "Ying and yang" Of a relevant protein. Brain Res Rev 61: 170-184.

Nicot, S. et al. 2013. L-type bovine spongiform encephalopathy in genetically susceptible and resistant sheep: Changes in prion strain or phenotypic plasticity of the disease-associated prion protein? J Infect Dis 209: 950-959.

Nichols, T. A. et al. 2009. Detection of protease-resistant cervid prion protein in water from a cwd-endemic area. Prion 3: 171-183.

Nicholson, E. M., B. W. Brunelle, J. A. Richt, M. E. Kehrli, Jr., and J. J. Greenlee. 2008. Identification of a heritable polymorphism in bovine prnp associated with genetic transmissible spongiform encephalopathy: Evidence of heritable bse. PLoS One 3: e2912.

Nilsson, M. R. 2004. Techniques to study amyloid fibril formation in vitro. Methods 34: 151-160.

Nodelijk, G. et al. 2011. Breeding with resistant rams leads to rapid control of classical scrapie in affected sheep flocks. Vet Res 42: 5.

Nuvolone, M., A. Aguzzi, and M. Heikenwalder. 2009. Cells and prions: A license to replicate. FEBS Lett 583: 2674-2684.

Okada, H. et al. 2014. The presence of disease-associated prion protein in skeletal muscle of cattle infected with classical bovine spongiform encephalopathy. J Vet Med Sci 76: 103-107.

Ono, F. et al. 2011. Atypical l-type bovine spongiform encephalopathy (l-bse) transmission to cynomolgus macaques, a non-human primate. Jpn J Infect Dis 64: 81-84.

Padilla, D. et al. 2011. Sheep and goat bse propagate more efficiently than cattle bse in human prp transgenic mice. PLoS Pathog 7: e1001319.

Palmer, M. S., A. J. Dryden, J. T. Hughes, and J. Collinge. 1991. Homozygous prion protein genotype predisposes to sporadic creutzfeldt- jakob disease. Nature 352: 340-342.

Paludi, D. et al. 2007. Different structural stability and toxicity of prp(arr) and prp(arq) sheep prion protein variants. J Neurochem 103: 2291-2300.

Pan, K. M. et al. 1993. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci U S A 90: 10962-10966.

Pan, Y. et al. 2006. Cellular prion protein promotes invasion and metastasis of gastric cancer. Faseb J.

Papasavva-Stylianou, P. et al. 2011. Prp gene polymorphisms in cyprus goats and their association with resistance or susceptibility to natural scrapie. Vet J.

Parchi, P. et al. 1996. Molecular basis of phenotypic variability in sporadic creutzfeldt-jakob disease. Ann Neurol 39: 767-778.

Parchi, P. et al. 1998. Different patterns of truncated prion protein fragments correlate with distinct phenotypes in p102l gerstmann-straussler-scheinker disease. Proc Natl Acad Sci U S A 95: 8322-8327.

Parchi, P. et al. 1999. Classification of sporadic creutzfeldt-jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol 46: 224-233.

Parchi, P., R. Strammiello, A. Giese, and H. Kretzschmar. 2011. Phenotypic variability of sporadic human prion disease and its molecular basis: Past, present, and future. Acta Neuropathol 121: 91-112.

Parizek, P. et al. 2001. Similar turnover and shedding of the cellular prion protein in primary lymphoid and neuronal cells. J Biol Chem 276: 44627-44632.

Parkin, E. T. et al. 2007. Cellular prion protein regulates beta-secretase cleavage of the alzheimer's amyloid precursor protein. Proc Natl Acad Sci U S A 104: 11062-11067.

Parsell, D. A., and S. Lindquist. 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu Rev Genet 27: 437-496.

Passet, B. et al. 2012. Prion protein and shadoo are involved in overlapping embryonic pathways and trophoblastic development. PLoS One 7: e41959.

Pastrana, M. A. et al. 2006. Isolation and characterization of a proteinase k-sensitive prp(sc) fraction. Biochemistry 45: 15710-15717.

Pattison, I. H. 1965. Scrapie in the welsh mountain breed of sheep and its experimental transmission to goats. Vet Rec 77: 1388-1390.

Pattison, I. H., W. S. Gordon, and G. C. Millson. 1959. Experimental production of scrapie in goats. J Comp Pathol 69: 300-312.

Pattison, I. H., and G. C. Millson. 1961. Scrapie produced experimentally in goats with special reference to the clinical syndrome. J Comp Pathol 71: 101-109.

Peretz, D. et al. 2002. A change in the conformation of prions accompanies the emergence of a new prion strain. Neuron 34: 921-932.

Peretz, D. et al. 1997. A conformational transition at the n terminus of the prion protein features in formation of the scrapie isoform. J Mol Biol 273: 614-622.

Perrier, V. et al. 2002. Dominant-negative inhibition of prion replication in transgenic mice. Proc Natl Acad Sci U S A 99: 13079-13084.

Peters, P. J. et al. 2003. Trafficking of prion proteins through a caveolae-mediated endosomal pathway. J Cell Biol 162: 703-717.

Petit, C. S., L. Besnier, E. Morel, M. Rousset, and S. Thenet. 2013. Roles of the cellular prion protein in the regulation of cell-cell junctions and barrier function. Tissue Barriers 1: e24377.

Piccardo, P. et al. 1998. Phenotypic variability of gerstmann-straussler-scheinker disease is associated with prion protein heterogeneity. J Neuropathol Exp Neurol 57: 979-988.

Pirisinu, L. et al. 2010. A new method for the characterization of strain-specific conformational stability of protease-sensitive and protease-resistant prp. PLoS One 5: e12723.

Pirisinu, L. et al. 2013. Small ruminant nor98 prions share biochemical features with human gerstmann-straussler-scheinker disease and variably protease-sensitive prionopathy. PLoS One 8: e66405.

Plinston, C. et al. 2011. Increased susceptibility of human-prp transgenic mice to bovine spongiform encephalopathy infection following passage in sheep. J Virol 85: 1174-1181.

Prestori, F. et al. 2008. Altered neuron excitability and synaptic plasticity in the cerebellar granular layer of juvenile prion protein knock-out mice with impaired motor control. J Neurosci 28: 7091-7103.

Priem, J. et al. 2014. Enhanced virulence of sheep-passaged bovine spongiform encephalopathy agent is revealed by decreased polymorphism barriers in prion protein conversion studies. J Virol 88: 2903-2912.

Pruden, S. J., M. M. McAllister, P. C. Schultheiss, D. O'Toole, and D. E. Christensen. 2004. Abomasal emptying defect of sheep may be an acquired form of dysautonomia. Vet Pathol 41: 164-169.

Prusiner, S. B. 1991. Molecular biology of prion diseases. Science 252: 1515-1522.

Prusiner, S. B. 1998a. The prion diseases. Brain Pathol 8: 499-513.

Prusiner, S. B. 1998b. Prions. Proc Natl Acad Sci U S A 95: 13363-13383.

Prusiner, S. B. 2004. Detecting mad cow disease. Sci Am 291: 86-93.

Prusiner, S. B. et al. 1993. Ablation of the prion protein (prp) gene in mice prevents scrapie and facilitates production of anti-prp antibodies. Proc Natl Acad Sci U S A 90: 10608-10612.

Prusiner, S. B. et al. 1981. Scrapie agent contains a hydrophobic protein. Proc Natl Acad Sci U S A 78: 6675-6679.

Prusiner, S. B. et al. 1990. Transgenetic studies implicate interactions between homologous prp isoforms in scrapie prion replication. Cell 63: 673-686.

Race, B., K. Meade-White, R. Race, and B. Chesebro. 2009a. Prion infectivity in fat of deer with chronic wasting disease. J Virol 83: 9608-9610.

Race, B. et al. 2009b. Susceptibilities of nonhuman primates to chronic wasting disease. Emerg Infect Dis 15: 1366-1376.

Rajendran, L., V. Udayar, and Z. V. Goodger. 2012. Lipid-anchored drugs for delivery into subcellular compartments. Trends Pharmacol Sci 33: 215-222.

Requena, J. R., and H. Wille. 2014. The structure of the infectious prion protein: Experimental data and molecular models. Prion 8.

Rezaei, H. et al. 2002. Amyloidogenic unfolding intermediates differentiate sheep prion protein variants. J Mol Biol 322: 799-814.

Richt, J. A., and S. M. Hall. 2008. Bse case associated with prion protein gene mutation. PLoS Pathog 4: e1000156.

Riek, R. et al. 1996. Nmr structure of the mouse prion protein domain prp(121-321). Nature 382: 180-182.

Riek, R. et al. 1998. Prion protein nmr structure and familial human spongiform encephalopathies. Proc Natl Acad Sci U S A 95: 11667-11672.

Rigter, A., and A. Bossers. 2005. Sheep scrapie susceptibility-linked polymorphisms do not modulate the initial binding of cellular to disease-associated prion protein prior to conversion. J Gen Virol 86: 2627-2634.

Saa, P. et al. 2012. Strain-specific role of rnas in prion replication. J Virol 86: 10494-10504.

Saborio, G. P., B. Permanne, and C. Soto. 2001. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 411: 810-813.

Saeki, K., Y. Matsumoto, and T. Onodera. 1996. Identification of a promoter region in the rat prion protein gene. Biochem Biophys Res Commun 219: 47-52.

Safar, J. 1996. The folding intermediate concept of prion protein formation and conformational links to infectivity. Curr Top Microbiol Immunol 207: 69-76.

Safar, J. et al. 1998. Eight prion strains have prp(sc) molecules with different conformations. Nat Med 4: 1157-1165.

Safar, J. G. et al. 2005. Diagnosis of human prion disease. Proc Natl Acad Sci U S A 102: 3501-3506.

Safar, J. G. et al. 2008. Transmission and detection of prions in feces. J Infect Dis 198: 81-89.

Saunders, S. E., S. L. Bartelt-Hunt, and J. C. Bartz. 2008. Prions in the environment: Occurrence, fate and mitigation. Prion 2: 162-169.

Saunders, S. E., S. L. Bartelt-Hunt, and J. C. Bartz. 2012a. Occurrence, transmission, and zoonotic potential of chronic wasting disease. Emerg Infect Dis 18: 369-376.

Saunders, S. E., J. C. Bartz, and S. L. Bartelt-Hunt. 2012b. Soil-mediated prion transmission: Is local soil-type a key determinant of prion disease incidence? Chemosphere 87: 661-667.

Saunders, S. E., R. A. Shikiya, K. Langenfeld, S. L. Bartelt-Hunt, and J. C. Bartz. 2011. Replication efficiency of soil-bound prions varies with soil type. J Virol 85: 5476-5482.

Scott, J. R., J. D. Foster, and H. Fraser. 1993a. Conjunctival instillation of scrapie in mice can produce disease. Vet Microbiol 34: 305-309.

Scott, M. et al. 1989. Transgenic mice expressing hamster prion protein produce species- specific scrapie infectivity and amyloid plaques. Cell 59: 847-857.

Scott, M. et al. 1993b. Propagation of prions with artificial properties in transgenic mice expressing chimeric prp genes. Cell 73: 979-988.

Scott, M. R., R. Kohler, D. Foster, and S. B. Prusiner. 1992. Chimeric prion protein expression in cultured cells and transgenic mice. Protein Sci 1: 986-997.

Scott, M. R., D. Peretz, H. O. Nguyen, S. J. Dearmond, and S. B. Prusiner. 2005. Transmission barriers for bovine, ovine, and human prions in transgenic mice. J Virol 79: 5259-5271.

Scott, M. R. et al. 1999. Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. Proc Natl Acad Sci U S A 96: 15137-15142.

Scheibel, T., and S. L. Lindquist. 2001. The role of conformational flexibility in prion propagation and maintenance for sup35p. Nat Struct Biol 8: 958-962.

Schmerr, M. J., A. Jenny, and R. C. Cutlip. 1997. Use of capillary sodium dodecyl sulfate gel electrophoresis to detect the prion protein extracted from scrapie-infected sheep. J Chromatogr B Biomed Sci Appl 697: 223-229.

Seelig, D. M., G. L. Mason, G. C. Telling, and E. A. Hoover. 2010. Pathogenesis of chronic wasting disease in cervidized transgenic mice. Am J Pathol 176: 2785-2797.

Serio, T. R. et al. 2000. Nucleated conformational conversion and the replication of conformational information by a prion determinant. Science 289: 1317-1321.

Shibuya, S., J. Higuchi, R. W. Shin, J. Tateishi, and T. Kitamoto. 1998. Codon 219 lys allele of prnp is not found in sporadic creutzfeldt-jakob disease. Ann Neurol 43: 826-828.

Shyng, S. L., J. E. Heuser, and D. A. Harris. 1994. A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. J Cell Biol 125: 1239-1250.

Shyng, S. L., M. T. Huber, and D. A. Harris. 1993. A prion protein cycles between the cell surface and an endocytic compartment in cultured neuroblastoma cells. J Biol Chem 268: 15922-15928.

Sibbald, B. 2004. Uk patient first to contract vcjd via blood transfusion. Cmaj 170: 1087.

Sigurdson, C. J. 2008. A prion disease of cervids: Chronic wasting disease. Vet Res 39: 41.

Sigurdson, C. J. et al. 2011. Spongiform encephalopathy in transgenic mice expressing a point mutation in the beta2-alpha2 loop of the prion protein. J Neurosci 31: 13840-13847.

Sigurdson, C. J. et al. 2006. Strain fidelity of chronic wasting disease upon murine adaptation. J Virol 80: 12303-12311.

Sigurdson, C. J., and M. W. Miller. 2003. Other animal prion diseases. Br Med Bull 66: 199-212.

Sigurdson, C. J. et al. 2010. A molecular switch controls interspecies prion disease transmission in mice. J Clin Invest 120: 2590-2599.

Sigurdson, C. J. et al. 2007. Prion strain discrimination using luminescent conjugated polymers. Nat Methods 4: 1023-1030.

Simmons, M. M. et al. 1996. Bse in great britain: Consistency of the neurohistopathological findings in two random annual samples of clinically suspect cases. Vet Rec 138: 175-177.

Simmons, M. M. et al. 2007. Experimental transmission of atypical scrapie to sheep. BMC Vet Res 3: 20.

Simmons, M. M. et al. 2011. Experimental oral transmission of atypical scrapie to sheep. Emerg Infect Dis 17: 848-854.

Simonic, T. et al. 2000. Cdna cloning of turtle prion protein. FEBS Lett 469: 33-38.

Siso, S. et al. 2010. Characterization of strains of ovine transmissible spongiform encephalopathy with a short prp(d) profiling method. J Comp Pathol.

Smith, C. B., C. J. Booth, and J. A. Pedersen. 2011. Fate of prions in soil: A review. J Environ Qual 40: 449-461.

Soldevila, M. et al. 2003. Prion susceptibility and protective alleles exhibit marked geographic differences. Hum Mutat 22: 104-105.

Sparkes, R. S. et al. 1986. Assignment of the human and mouse prion protein genes to homologous chromosomes. Proc Natl Acad Sci U S A 83: 7358-7362.

Spencer, M. D., R. S. Knight, and R. G. Will. 2002. First hundred cases of variant creutzfeldt-jakob disease: Retrospective case note review of early psychiatric and neurological features. BMJ 324: 1479-1482.

Spiropoulos, J., S. A. Hawkins, M. M. Simmons, and S. J. Bellworthy. 2014. Evidence of in utero transmission of classical scrapie in sheep. J Virol 88: 4591-4594.

Spraker, T. R. et al. 2010. Detection of the abnormal isoform of the prion protein associated with chronic wasting disease in the optic pathways of the brain and retina of rocky mountain elk (cervus elaphus nelsoni). Vet Pathol 47: 536-546.

Stack, M. J. et al. 2011. Experimental bovine spongiform encephalopathy: Detection of prp(sc) in the small intestine relative to exposure dose and age. J Comp Pathol 145: 289-301.

Stahl, N., D. R. Borchelt, K. Hsiao, and S. B. Prusiner. 1987. Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell 51: 229-240.

Steele, A. D., S. Lindquist, and A. Aguzzi. 2007. The prion protein knockout mouse: A phenotype under challenge. Prion 1: 83-93.

Strumbo, B., S. Ronchi, L. C. Bolis, and T. Simonic. 2001. Molecular cloning of the cdna coding for xenopus laevis prion protein. FEBS Lett 508: 170-174.

Sunyach, C. et al. 2003. The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. Embo J 22: 3591-3601.

Swietnicki, W., R. B. Petersen, P. Gambetti, and W. K. Surewicz. 1998. Familial mutations and the thermodynamic stability of the recombinant human prion protein. J Biol Chem 273: 31048-31052.

Tamguney, G. et al. 2006. Transmission of elk and deer prions to transgenic mice. J Virol 80: 9104-9114.

Tamguney, G. et al. 2009. Asymptomatic deer excrete infectious prions in faeces. Nature 461: 529-532.

Tamguney, G. et al. 2012. Salivary prions in sheep and deer. Prion 6: 52-61.

Tan, B. C. et al. 2012. Significant differences in incubation times in sheep infected with bovine spongiform encephalopathy result from variation at codon 141 in the prnp gene. J Gen Virol 93: 2749-2756.

Tanaka, M., S. R. Collins, B. H. Toyama, and J. S. Weissman. 2006. The physical basis of how prion conformations determine strain phenotypes. Nature 442: 585-589.

Tanaka, M., P. Chien, N. Naber, R. Cooke, and J. S. Weissman. 2004. Conformational variations in an infectious protein determine prion strain differences. Nature 428: 323-328.

Taraboulos, A., A. J. Raeber, D. R. Borchelt, D. Serban, and S. B. Prusiner. 1992. Synthesis and trafficking of prion proteins in cultured cells. Mol Biol Cell 3: 851-863.

Taylor, D. M., C. E. Ferguson, C. J. Bostock, and M. Dawson. 1995. Absence of disease in mice receiving milk from cows with bovine spongiform encephalopathy. Vet Rec 136: 592.

Taylor, D. M., I. McConnell, and H. Fraser. 1996. Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. J Gen Virol 77: 1595-1599.

Telling, G. C. et al. 1994. Transmission of creutzfeldt-jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. Proc Natl Acad Sci U S A 91: 9936-9940.

Telling, G. C. et al. 1995. Prion propagation in mice expressing human and chimeric prp transgenes implicates the interaction of cellular prp with another protein. Cell 83: 79-90.

Terry, L. A. et al. 2003. Detection of disease-specific prp in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy. Vet Rec 152: 387-392.

Thackray, A. M., L. Hopkins, R. Lockey, J. Spiropoulos, and R. Bujdoso. 2011. Emergence of multiple prion strains from single isolates of ovine scrapie. J Gen Virol 92: 1482-1491.

Thackray, A. M., L. Hopkins, J. Spiropoulos, and R. Bujdoso. 2008. Molecular and transmission characteristics of primary-passaged ovine scrapie isolates in conventional and ovine prp transgenic mice. J Virol 82: 11197-11207.

Thomzig, A. et al. 2007. Accumulation of pathological prion protein prpsc in the skin of animals with experimental and natural scrapie. PLoS Pathog 3: e66.

Thorgeirsdottir, S., S. Sigurdarson, H. M. Thorisson, G. Georgsson, and A. Palsdottir. 1999. Prp gene polymorphism and natural scrapie in icelandic sheep. J Gen Virol 80 ( Pt 9): 2527-2534.

Thuring, C. M. et al. 2005. Immunohistochemical distinction between preclinical bovine spongiform encephalopathy and scrapie infection in sheep. J Comp Pathol 132: 59-69.

Torres, J. M. et al. 2011. Classical bovine spongiform encephalopathy by transmission of h-type prion in homologous prion protein context. Emerg Infect Dis 17: 1636-1644.

Torres, J. M. et al. 2013. Spontaneous generation of infectious prion disease in transgenic mice. Emerg Infect Dis 19: 1938-1947.

Torres, J. M. et al. 2014. Elements modulating the prion species barrier and its passage consequences. PLoS One 9: e89722.

Tremblay, P. et al. 2004. Mutant prpsc conformers induced by a synthetic peptide and several prion strains. J Virol 78: 2088-2099.

Tveit, H. et al. 2005. Proteolytic processing of the ovine prion protein in cell cultures. Biochem Biophys Res Commun.

Tzaban, S. et al. 2002. Protease-sensitive scrapie prion protein in aggregates of heterogeneous sizes. Biochemistry 41: 12868-12875.

Vaccari, G. et al. 2007. Prion protein alleles showing protective effect on the susceptibility of sheep to scrapie and bse. J Virol.

Vaccari, G. et al. 2006. Identification of an allelic variant of the goat prp gene associated with resistance to scrapie. J Gen Virol 87: 1395-1402.

Vaccari, G. et al. 2009a. State-of-the-art review of goat tse in the european union, with special emphasis on prnp genetics and epidemiology. Vet Res 40: 48.

Vaccari, G. et al. 2001. Prp genotype in sarda breed sheep and its relevance to scrapie. Brief report. Arch Virol 146: 2029-2037.

Vaccari, G. et al. 2009b. Protective effect of the at137rq and arqk176 prp allele against classical scrapie in sarda breed sheep. Vet Res 40: 19.

van Keulen, L. J., A. Bossers, and F. van Zijderveld. 2008a. Tse pathogenesis in cattle and sheep. Vet Res 39: 24.

van Keulen, L. J. et al. 1996. Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. J Clin Microbiol 34: 1228-1231.

van Keulen, L. J., M. E. Vromans, C. H. Dolstra, A. Bossers, and F. G. van Zijderveld. 2008b. Pathogenesis of bovine spongiform encephalopathy in sheep. Arch Virol 153: 445-453.

van Keulen, L. J., M. E. Vromans, and F. G. van Zijderveld. 2002. Early and late pathogenesis of natural scrapie infection in sheep. Apmis 110: 23-32.

Vey, M. et al. 1996. Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proc Natl Acad Sci U S A 93: 14945-14949.

Vincent, B. et al. 2000. Phorbol ester-regulated cleavage of normal prion protein in hek293 human cells and murine neurons. J Biol Chem 275: 35612-35616.

Vincent, B. et al. 2001. The disintegrins adam10 and tace contribute to the constitutive and phorbol ester-regulated normal cleavage of the cellular prion protein. J Biol Chem 276: 37743-37746.

Wadia, J. S., M. Schaller, R. A. Williamson, and S. F. Dowdy. 2008. Pathologic prion protein infects cells by lipid-raft dependent macropinocytosis. PLoS One 3: e3314.

Wadsworth, J. D. et al. 2004. Human prion protein with valine 129 prevents expression of variant cjd phenotype. Science 306: 1793-1796.

Wadsworth, J. D. et al. 1999. Strain-specific prion-protein conformation determined by metal ions. Nat Cell Biol 1: 55-59.

Wadsworth, J. D. et al. 2013. Atypical scrapie prions from sheep and lack of disease in transgenic mice overexpressing human prion protein. Emerg Infect Dis 19: 1731-1739.

Wang, F., X. Wang, C. G. Yuan, and J. Ma. 2010. Generating a prion with bacterially expressed recombinant prion protein. Science 327: 1132-1135.

Watt, N. T., and N. M. Hooper. 2005. Reactive oxygen species (ros)-mediated beta-cleavage of the prion protein in the mechanism of the cellular response to oxidative stress. Biochem Soc Trans 33: 1123-1125.

Watts, J. C. et al. 2007. The cns glycoprotein shadoo has prp(c)-like protective properties and displays reduced levels in prion infections. EMBO J 26: 4038-4050.

Watts, J. C. et al. 2014. Evidence that bank vole prp is a universal acceptor for prions. PLoS Pathog 10: e1003990.

Wells, G. A. et al. 1994. Infectivity in the ileum of cattle challenged orally with bovine spongiform encephalopathy. Vet Rec 135: 40-41.

Wells, G. A. et al. 2003. Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs. J Gen Virol 84: 1021-1031.

Wells, G. A. et al. 1998. Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (bse): An update. Vet Rec 142: 103-106.

Wells, G. A. et al. 1987. A novel progressive spongiform encephalopathy in cattle. Vet Rec 121: 419-420.

Wells, G. A., J. Spiropoulos, S. A. Hawkins, and S. J. Ryder. 2005. Pathogenesis of experimental bovine spongiform encephalopathy: Preclinical infectivity in tonsil and observations on the distribution of lingual tonsil in slaughtered cattle. Vet Rec 156: 401-407.

Westaway, D. et al. 1994. Structure and polymorphism of the mouse prion protein gene. Proc Natl Acad Sci U S A 91: 6418-6422.

Wilesmith, J. W., J. B. Ryan, and M. J. Atkinson. 1991. Bovine spongiform encephalopathy: Epidemiological studies on the origin. Vet Rec 128: 199-203.

Wilson, R. et al. 2012. Chronic wasting disease and atypical forms of bovine spongiform encephalopathy and scrapie are not transmissible to mice expressing wild-type levels of human prion protein. J Gen Virol 93: 1624-1629.

Will, R. G. 2003. Acquired prion disease: Iatrogenic cjd, variant cjd, kuru. Br Med Bull 66: 255-265.

Will, R. G. et al. 1996. A new variant of creutzfeldt-jakob disease in the uk. Lancet 347: 921-925.

Williams, E. S., and S. Young. 1980. Chronic wasting disease of captive mule deer: A spongiform encephalopathy. J Wildl Dis 16: 89-98.

Wong, C. et al. 2001. Sulfated glycans and elevated temperature stimulate prp(sc)-dependent cell-free formation of protease-resistant prion protein. Embo J 20: 377-386.

Wrathall, A. E. et al. 2002. Studies of embryo transfer from cattle clinically affected by bovine spongiform encephalopathy (bse). Vet Rec 150: 365-378.

Wroe, S. J. et al. 2006. Clinical presentation and pre-mortem diagnosis of variant creutzfeldt-jakob disease associated with blood transfusion: A case report. Lancet 368: 2061-2067.

Young, R. et al. 2009. The prion or the related shadoo protein is required for early mouse embryogenesis. FEBS Lett 583: 3296-3300.

Yull, H. M. et al. 2006. Detection of type 1 prion protein in variant creutzfeldt-jakob disease. Am J Pathol 168: 151-157.

Zanusso, G. et al. 2001. Ph-dependent prion protein conformation in classical creutzfeldt-jakob disease. J Biol Chem 276: 40377-40380.

Zeidler, M., G. Stewart, S. N. Cousens, K. Estibeiro, and R. G. Will. 1997. Codon 129 genotype and new variant cjd. Lancet 350: 668.

Zhang, C. C., A. D. Steele, S. Lindquist, and H. F. Lodish. 2006. Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. Proc Natl Acad Sci U S A.

Zhang, Y., W. Swietnicki, M. G. Zagorski, W. K. Surewicz, and F. D. Sonnichsen. 2000. Solution structure of the e200k variant of human prion protein. Implications for the mechanism of pathogenesis in familial prion diseases. J Biol Chem 275: 33650-33654.

Zou, W. Q. et al. 2003. Identification of novel proteinase k-resistant c-terminal fragments of prp in creutzfeldt-jakob disease. J Biol Chem 278: 40429-40436.

# XI. PUBLICATIONS

1. **Patricia Aguilar-Calvo**, Juan Carlos Espinosa, Belén Pintado, Olivier Andréoletti and Juan María Torres. $K_{176}$-$PrP^C$ polymorphic variant provides resistance to classical scrapie and BSE but not atypical scrapie. In preparation.
2. **Patricia Aguilar-Calvo**, Juan Carlos Espinosa, Belén Pintado, Olivier Andréoletti and Juan María Torres.Transgenic mice expressing the $R_{171}$-$PrP^C$ variant confirm the resistance of this genotype to classical scrapie infection but not to atypical scrapie or BSE. In preparation.
3. **Patricia Aguilar-Calvo**, Christine Fast, Juan-Carlos Espinosa, Martin H Groschup, Wilfred Goldmann, Alex Bosser and Juan-María Torres. Effect of $Q_{211}$ and $K_{222}$ *prnp* polymorphic variants in the susceptibility of goats to the oral infection with goat-BSE. Submitted.
4. **Patricia Aguilar-Calvo**, Juan Carlos Espinosa, Belén Pintado, Alfonso Gutiérrez-Adán, Elia Alamillo, Olivier Andreoletti and Juan María Torres. Role of goat $M_{142}$-$PrP^C$ polymorphic variant in resistance to prion agents. In preparation.
5. **Patricia Aguilar-Calvo**, Consolación García, Juan Carlos Espinosa, Olivier Andreoletti and Juan María Torres. Prion and Prion-like diseases in animals. Accepted in Virus Research.
6. Silvio Notari, Xiangzhu Xiao, Juan Carlos Espinosa, Yvonne Cohen, Qing Liuting, **Patricia Aguilar-Calvo**, Diane Kofskey, Ignazio Cali, Laura Cracco, Qingzhong Kong, Juan Maria Torres, Wen-Quan Zou and Pierluigi Gambetti. Transmissibility characteristics of variably protease sensitive prionopathy. Accepted in Emerging Infectious Diseases.
7. Juan-Maria Torres, Juan-Carlos Espinosa, **Patricia Aguilar-Calvo**, María-Eugenia Herva, Aroa Relaño-Ginés, Ana Villa-Diaz, Mónica Morales, Beatriz Parra, Elia Alamillo, Alejandro Brun, Joaquín Castilla, Susana Molina, Steve AC Hawkins and Olivier Andreoletti. Elements modulating the prion species barrier and its passage consequences. *Plos ONE*. 2014 Mar. doi: 10.1371/journal.pone.0089722.
8. **Patricia Aguilar-Calvo P,** Juan-Carlos Espinosa, Belén Pintado, Alfonso Gutiérrez-Adán, Elia Alamillo, Alberto Miranda, Irene Prieto, Alex Bossers, Olivier Andreoletti and Juan-María Torres. Role of the goat $K_{222}$-$PrP^C$ polymorphic variant in prion infection resistance. *Journal of virology*. 2014 Mar;88(5):2670-6. doi: 10.1128/JVI.02074-13.
9. Caroline Lacroux, Cécile Perrin-Chauvineau, Fabien Corbière, Naima Aron, **Patricia Aguilar-Calvo**, Juan-María Torres, Pierrette Costes, Isabelle Brémaud, Séverine Lugan, François Schelcher, Francis Barillet and Olivier Andréoletti. Genetic resistance to Scrapie infection in experimentally

challenged goats. *Journal of virology*. 2014 Mar;88(5):2406-13. doi: 10.1128/JVI.02872-13.

10. Juan-María Torres, Joaquín Castilla, Belén Pintado, Alfonso Gutiérrez-Adan, Olivier Andréoletti, **Patricia Aguilar-Calvo**, Ana-Isabel Arroba, Beatriz Parra-Arrondo, Isidre Ferrer, Jorge Manzanares and Juan-Carlos Espinosa. Spontaneous generation of infectious prion disease in transgenic mice. *Emerging Infectious Disease*. 2013 Dec;19(12):1938-47. doi: 10.3201/eid1912.130106.