Influence of base-excision-repair pathway enzymes on prion pathogenesis

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Dette har jeg aldri gjort før

Så det klarer jeg sikkert

-Pippi Langstrømpe

Til mamma og pappa,

Lisbeth og Svein Osnes.

Table of Contents

Preface

The present work is a result of a collaboration between three laboratories located in different institutions. The Section of Biochemistry and Physiology, Department of Basic Sciences and Aquatic Medicine, at the Norwegian University of Life Sciences, which is "my" lab, has initiated and been the main driver behind the studies.

The inoculation experiments and sampling of material were done at the Norwegian Veterinary Institute in Oslo, under supervision of Sylvie L. Benestad.

Analysis of DNA damage and epigenetic alterations as well as RNA sequencing and mitochondrial activity analysis were performed in Magnar Bjørås' laboratory at the Norwegian University of Science and Technology in Trondheim. The specific lines of mice used in my studies were made available from Bjørås' laboratory.

I would like to start by acknowledging my PhD committee for evaluating this thesis.

This work would not have been accomplished without guide and support from several people.

First, I would like to thank my main supervisor, Michael A. Tranulis. Thank you for giving me this opportunity and for being so enthusiastic and supportive, and for never losing patience with me. Your door has always been open and you have constantly been available for discussions and questions. I will be forever grateful for all support and great discussions especially during writing of the thesis.

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Finally, I want to thank my husband, Jens, for your endless patience and love, and for being there when I needed it most. Jacob, Emma and Hannah, thank you for bringing love, joy and happiness into my life every day and for reminding me of what is most important in life. I love you to the moon and back.

Abstract

Experimental prion disease in mice is a well-established model system for studying the pathogenesis of prion-induced neurodegeneration. The mouse-adapted Rocky Mountain Laboratories (RML) strain is commonly used for this purpose.

With the ever-increasing catalogue of transgenic mice in which one or more genes have been invalidated, a considerable number of studies of prion disease have been performed in knockout lines, in search for genes that could influence the disease progression and presentation. The general idea behind such studies is to gain knowledge of the pathogenesis and to identify new targets for disease treatment and/or prevention. This is indeed also the foundation of the studies in this thesis.

We have asked the question; do DNA repair enzymes belonging to the base-excision-repair (BER) pathway contribute cellular protection against prion-induced toxicity? To explore this, we have used three relatively newly developed lines of BER-enzyme knockouts. Although some of the physiological roles of the individual BER enzymes have been established, the full spectrum of functions are still very much under investigation. Thus, the studies in this thesis have also had this aspect in mind—namely that experimental prion disease in these mouse models could potentially reveal less explored functions of these enzymes.

Three lines of transgenic mice with compromised BER-enzyme activity were subjected to RML prion disease, and in two of our studies (Paper II and III) samples were analyzed both at onset and end-stage of disease. The most striking observations from these studies are that the pre-clinical progression of experimental prion disease appears largely unaffected by BER-enzyme activities. However, loss of BER enzymes in all three models used resulted in a more dramatic and shortened clinical (toxic) phase of the disease, suggesting that BER-enzyme activity in various ways contributes neuronal protection in the final clinical phase.

Taken together, one could, based upon our results, reach the conclusion that BER repair of oxidative DNA damage in prion disease is of moderate or minor importance. It should, however, be noted that the model we have used, with intracerebral inoculation of RML prions, is a brutally efficient disease model, resulting in a disease progression that barely is affected by any genetic or other intervention. When taking

this aspect into consideration, our observations of significantly shortened clinical duration in the absence of different BER activities, to my mind, indicates that these DNA repair enzymes play a significant part in anti-prion neuroprotection.

Sammendrag (Summary in Norwegian)

Det finnes flere alvorlige hjernesykdommer som med en fellesbetegnelse kalles kroniske nevrodegenerative sykdommer. De fleste av disse har en langsom utvikling og de fører til at nerveceller ubønnhørlig dør i de delene av hjernen som rammes. De mest kjente er Alzheimers sykdom (AD), Parkinsons sykdom, Huntingtons sykdom og prionsykdommene. AD er mest vanlig, og i 2006 var det omlag 26,6 millioner mennesker som led av sykdommen på verdensbasis. Med stadig aldrende populasjon, antas det at mere enn én prosent av verdens befolkning vil være rammet av sykdommen i 2050.

Et fellestrekk for sykdomsgruppen er opphopning av feilfoldede og skadelige former av kroppsegne proteiner. Hva som forårsaker dette og de nøyaktige mekanismene bak er fortsatt ikke kjent. Ved prionsykdom blir det cellulære prionproteinet, PrP^{C}, feilfoldet til PrP^{Sc} (Scrapie). Det karakteristiske for prionsykdommene er opphopning av PrP^{Sc}, dannelse av vakuoler i og omkring nerveceller, proliferasjon av støtteceller og tap av nevroner. Alle symptomer stammer fra skadene i hjernen.

Prionsykdommer forekommer naturlig hos menneske i form av Creutzfeldt-Jakob sykdom og hos en rekke drøvtyggere, som skrapesyke hos sau og geit, kugalskap hos storfe og skrantesjuke hos hjortedyr; nå også påvist hos reinsdyr og elg i Norge i 2016.

PrP^{C} sin normalfunksjon er omdiskutert og fortsatt delvis ukjent, men flere forskere mener at PrP^{C} kan ha en beskyttende funksjon, spesielt på nerveceller, i møte med alvorlige belastninger. Når celler skades eller belastes slik at deres normale balanse forrykkes, sier vi gjerne at cellene utsettes for stress. Dette er vanlig og celler er derfor utstyrt med en rekke beskyttelses- og reparasjonsmekanismer. En hyppig forekommende form for cellulært stress forårsakes av oksygenradikaler (ROS) som består av svært reaktive og ustabile oksygenholdige molekyler som kan skade nukleinsyrer (DNA, RNA), proteiner og lipider i cellen. Dersom stressbelastningene nervecellene utsettes for overskrider deres beskyttelses- og reparasjonsevne, vil cellene svekkes og i verste fall dø. Ved nevrodegenerative sykdommer skjer dette med nervecellene, og man regner med at stress forårsaket av ROS er en viktig faktor.

Oksygenradikaler dannes kontinuerlig spesielt i mitokondriene, og har viktige cellulære funksjoner, men dersom nivået blir for høyt og langvarig vil skader kunne oppstå også i cellekjernen, hvor DNA rammes. Det finnes en rekke enzymer som reparerer DNA-skade forårsaket av oksygenradikaler. Disse DNA-reparasjonsenzymene har delvis overlappende funksjoner og danner tilsammen en reaksjonssekvens som kalles «base excision repair» (BER).

I denne avhandlingen har vi studert utvikling av eksperimentell prionsykdom hos tre forskjellige linjer av transgene mus, som mangler ett eller flere slike DNA-reparasjonsenzymer. Målet har vært å utrede hvilken rolle slik DNA-reparasjon har i beskyttelse mot de skadene som oppstår ved prionsykdom. Vi har studert mus som

ikke uttrykker DNA-glykosylasene *Ogg1/Mutyh*, *Neil2* og *Neil3*. Disse enzymene «skanner» DNA og gjenkjenner skadede (Ogg1, Neil2 og Neil3) eller feilparede (Mutyh) nukleotidbaser, som de vipper ut av DNA-heliksen og kutter N-glykosylbindingen mellom basen og deoksyribose, før andre enzymer fullfører reparasjonen.

Eksperimentell prionsykdom hos mus gjenskaper alle særtrekkene ved naturlig forekommende prionsykdom og er derfor meget verdifulle sykdomsmodeller. Sykdommen forløper i to hovedfaser. Den første fasen er uten symptomer og kan vare i flere måneder. I denne fasen akkumuleres PrP^{Sc} og støtteceller som astrocytter og mikroglia aktiveres og deler seg. Hvilken betydning mikroglia har i sykdomsutviklingen er omdiskutert. De fleste ser imidlertid ut til å mene at mikroglia primært har en beskyttende rolle ved prionsykdom, men at cellene mot slutten av sykdomsforløpet gjennom stimulering av betennelsesreaksjoner kan gjøre vondt verre. Den andre fasen i prionsykdom er den toksiske fasen. Den er kortere, med økning av mitokondriell og nukleær DNA-skade og tap av nevroner.

I artikkel I studerte vi transgene mus som manglet to DNA-reparasjonsenzymer, Ogg1 og Mutyh, og villtypemus etter inokulering av PrP^{Sc} intrakranialt. Vi så en lengre klinisk sykdomsfase hos villtypemusene, men patologien var lik i hjernen hos begge gruppene. Ved måling av DNA-skade i hjernen var det en signifikant forskjell mellom gruppene. Musene som mangler de spesifikke reparasjonsenzymene hadde en ekstrem økning i DNA-skade, som kan være årsaken til den kortere kliniske fasen.

I artikkel II studerte vi villtypemus og mus uten Neil3, *Neil* $3^{-/-}$. Også her levde villtypemusene noe lenger. Vi sammenliknet ekspresjonsnivået av mikrogliamarkørene *Cd68* og *Cd86* og betennelsesmarkørene *TNFα* og *Il1β*. Vi fant aktivering av mikroglia før klinisk sykdom og en ytterligere aktivering i den toksiske fasen, men det var ingen forskjell mellom gruppene. I tillegg ble det observert mindre gliose hos *Neil* $3^{-/-}$-musene. Villtypemus viste en tydelig oppregulering av Dcx^{+}, som er en nevroblastmarkør, mens markøren for modne neuroner, NeuN, sank betraktelig hos *Neil3*$^{-/-}$ -mus i forhold til villtypemusene i den toksiske fasen. Dette støtter betydningen av *Neil3* i aktiveringen av adult nevrogenese etter kraftig tap av nevroner i den kliniske fasen.

I artikkel III studerte vi villtypemus og mus uten Neil2, *Neil* $2^{-/-}$. Den første fasen av prionsykdommen forløp veldig likt mellom gruppene, men ved starten av kliniske symptomer døde *Neil* $2^{-/-}$-musene raskt. Mengden av DNA-skade var veldig lik mellom gruppene, med unntak av mye lavere nivå av DNA skade i milten hos *Neil* $2^{-/-}$-musene ved sluttfasen av sykdom. Funksjonen til mitokondriene, målt som aktiviteten til respirasjonskompleksene I og V i elektrontransportkjeden, ble undersøkt i både hjernen og milten. I den toksiske fasen observerte vi en kraftig økning av aktiviteten av begge kompleksene i milten hos *Neil* $2^{-/-}$-musene. Transkripsjonsanalyser av milt ble utført ved hjelp av RNA-sekvensering. Ved sluttstadiet av sykdommen var det store forskjeller mellom gruppene i genuttrykk.

Hos *Neil 2*$^{-/-}$-musene ble det funnet økt uttrykk i gener som stimulerer proliferasjon av immunceller, som igjen kan føre til en forsterket og skadelig betennelsesreaksjon. Dette kan være en av årsakene til at *Neil 2*$^{-/-}$musene døde raskere enn normalmusene.

Sett under ett viser våre studier at den prekliniske fasen av prionsykdom og sykdommens hovedtrekk ikke påvirkes kraftig av BER-enzymaktiviteter. Men i alle de tre musemodellene vi har benyttet ser vi at tap av BER-enzymer fører til at sykdommen får et mere aggressivt klinisk forløp, noe som viser at i denne fasen av sykdommen spiller BER-enzymer viktige roller i beskyttelsen av nervecellene.

Vi må også ta i betraktning at sykdomsmodellen vi har benyttet, med direkte injeksjon av RML-prioner i hjernen, er en brutalt effektiv modell, hvor forsøk har vist at sykdomsforløpet knapt påvirkes av genetiske eller andre faktorer. Sett i lys av dette er de signifikante effektene vi observerer i den kliniske fasen tegn på at BER-enzymer spiller viktige roller i beskyttelse av nerveceller mot prionindusert skade.

Abbreviations

5-OHC	5-hydroxycytosine
8-oxoG	7,8-dihydroxy-8-guanin
A	adenine
Aβ	amyloid beta
AD	Alzheimer's disease
AP	apurinic/apyrimidinic
APE1	AP endonuclease 1
APP	amyloid precursor protein
BER	base excision repair
BSE	bovine spongiform encephalopathy
BSE-H	High, based on the difference in PrP^{Sc} molecular masses identified after Western blot analysis
BSE-L	Low, based on the difference in PrP^{Sc} molecular masses identified after Western blot analysis
C	cytosine
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CWD	chronic wasting disease
DNA	deoxyribonucleic acid
DRM	detergent resistant membrane
DSB	double strand break
ER	endoplasmic reticulum
FCD	follicular dendritic cells
FFI	Fatal familial insomnia
FSE	Feline spongiform encephalopathy
G	guanine

GPI	glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Schenker
HD	Huntington's disease
Htt	huntingtin protein
kDa	kilo Dalton
LIG I	DNA ligase I
mHtt	mutated huntingtin protein
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
ND	neurodegenerative disease
Nei	endonuclease VIII
NEIL	Nei-like
NER	nucleotide excision repair
NLS	nuclear localization signals
OGG1	8-oxoG DNA glycosylase
PD	Parkinson's disease
PK	Proteinase K
Poly-Q	polyglutamine-rich version of the protein huntingtin
PRNP	prion protein gene
PrP KO	prion protein knockout mice
PrP^{c}	cellular prion protein
PrP^{Sc}	scrapie prion protein
Q	glutamine
ROS	reactive oxygen species
SFI	Sporadic fatal insomnia
SGZ	subgranule zone of the hippocampal dentate gyrus
Sha	Syrian hamsters

SOD	superoxide dismutase
SSB	DNA single-strand break
SVZ	subventricular zone of the lateral ventricle
T	thymine
TME	Transmissible mink encephalopathy
TNF-α	tumor necrosis factor alpha
XRCC1	X-ray repair cross complementing protein 1

List of papers

Paper I

Clara M.O. Jalland, Sylvie L. Benestad, Cecilie Ersdal, Katja Scheffler, Rajikala Suganthan, Yusaku Nakabeppu, Lars Eide, Magnar Bjørås, Michael A. Tranulis. (2014) Accelerated clinical course of prion disease in mice compromised in repair of oxidative DNA damage. **Free Radical Biology and Medicine** 68:1-7.

Paper II

Clara M.O. Jalland, Katja Scheffler, Sylvie L. Benestad, Torfinn Moldal, Cecilie Ersdal, Rajikala Suganthan, Gjermund Gunnes, Magnar Bjørås, Michael A. Tranulis. (2016) Neil3 induced neurogenesis protects against prion disease during the clinical phase. **Scientific Reports** 6:37844

Paper III

Katja Scheffler, Clara M.O. Jalland, Sylvie L. Benestad, Torfinn Moldal, Cecilie Ersdal, Rajikala Suganthan, Gjermund Gunnes, Michael A. Tranulis, Magnar Bjørås. DNA glycosylase Neil2 is protective during clinical prion disease. ***Manuscript.***

Introduction

Neurodegenerative diseases

Neurodegenerative diseases (NDs) are characterized by progressive neurological dysfunction, caused by neuronal death in defined areas of the brain, which leads to distinct clinical presentations and disease progression (Jellinger 2009). Although the occurrence and clinical symptoms differ vastly among these diseases, important aspects of their underlying pathologies overlap. One striking feature in many of these diseases is the prevalent accumulation of abnormally folded proteins, both inside neurons and in the extracellular milieu. Thus, NDs are often referred to as diseases of protein misfolding (Gregersen 2006) or simply proteinopathies (Forman, Trojanowski et al. 2004). Among the NDs, Alzheimer's disease (AD) is by far the most common, with a worldwide occurrence of about 26 million.

The incidence of degenerative brain diseases, such as AD, is expected to quadruple by 2050, because of the increasingly aged population. By 2050, it is estimated that 1 in 85 persons worldwide will be living with the disease. A high-level care, equivalent to that of a nursing home, will be needed for approximately 40-50 % of these patients (Brookmeyer, Johnson et al. 2007), making AD one of the most financially costly diseases (Meek, McKeithan et al. 1998, Bonin-Guillaume, Zekry et al. 2005). The total estimated costs of dementia worldwide were US$ 604 billion in 2010, or approximately 1 % of the world's gross domestic product (Wimo and Prince 2010). About 70 % of the costs occur in Northern America and Western Europe.

In order to develop strategies for prevention or treatment of neurodegenerative diseases associated with protein misfolding and aggregation, detailed information about the pathogenic mechanisms is a prerequisite. Comparative approaches can be beneficial as there are many common paths in the pathogenesis of the proteinopathies.

Neurodegeneration and protein aggregation.

As shown in Table 1, the identity and characteristics of protein aggregates vary among the brain proteinopathies.

Table 1. Neurodegenerative diseases characterized by deposits of misfolded, host-encoded proteins inside or in the surroundings of neurons

Disease	Native protein	Abnormal protein	Subcellular localization
Alzheimer's disease	APP	Amyloid-β	Mostly extracellular
	Tau	Hyper-phosphorylated Tau	Cytoplasmic
Parkinson's disease	α-Synuclein	α-Synuclein aggregates (Lewy bodies)	Cytoplasmic
Huntington's disease	Huntingtin (Htt)	mHtt with Poly-Q expansions	Intranuclear
Prion disease	PrP^{C}	PrP^{Sc}	Mostly extracellular

APP: amyloid precursor protein. Poly-Q: polyglutamine-rich version of the protein huntingtin.

In AD, large protein aggregates, referred to as senile plaques, are observed in the most heavily affected brain areas. These aggregates, which can be observed in the light microscope, consist of several protein species, but of fundamental importance is amyloid beta (Aβ). The Aβ fragments are derived from the amyloid precursor protein (APP), through proteolytic processing by a combination of the proteases gamma- and beta-secretase. The liberated fragments subsequently aggregate in the extracellular milieu, together with other components. In addition, aggregates of the microtubule-associated protein Tau are found inside neurons. These are known as neurofibrillary tangles and have been shown to consist of hyperphosphorylated conformers of Tau, reviewed in (Grundke-Iqbal, Iqbal et al. 1986, Selkoe 2004). Together with senile plaques, these are pathological hallmarks of AD. The normal function of Tau is to stabilize neuronal microtubuli. Upon hyperphosphorylation, the normally very soluble Tau protein converts into an aggregation-prone conformer, which self-assembles into filaments. This occurs not only in AD, but also in frontotemporal dementia and in other disease entities collectively known as “tauopathies” (Grundke-Iqbal, Iqbal et al.

1986, Spillantini, Goedert et al. 1997, Goedert and Spillantini 2006, Goedert, Clavaguera et al. 2010, Clavaguera, Lavenir et al. 2013).

In Parkinson's disease (PD), large cytoplasmic aggregates of the protein alpha-synuclein constitute the main component of so-called Lewy bodies. In PD, these aggregates lead to the loss of dopaminergic motor neurons, and this accounts for some of the most striking clinical symptoms, reviewed in (Schapira and Tolosa 2010, Goedert, Spillantini et al. 2013). Lewy body aggregates are also observed in other forms of dementia known as Lewy body dementia or synucleinopathies (Uversky 2007).

In Huntington's disease (HD), intranuclear deposits are found. One of the first descriptions of HD was by the county doctor Johan Christian Lund who, in 1860, noted that a peculiar form of dementia characterized by jerking movements was particularly prevalent in the mountain valley of Setesdal, Norway. The intranuclear deposits consist of huntingtin protein (Htt) with so-called CAG triplet repeat expansion. Since the triplet CAG encodes the amino acid glutamine (Q), these expansions are commonly known as poly-Q expansions (Lee, Lim et al. 2011). The normal function of Htt is not clarified. The protein is expressed in most cells, but at particularly high levels in brain and testes, which is also the case for the prion protein. Mutated Htt, known as mHtt is a classic example of "gain-of-toxicity" mode of pathogenesis. I will briefly discuss the "loss-of-function" versus "gain-of-toxicity" modes of pathogenesis in prion disease later in the thesis.

In prion disease, the cellular prion protein (PrP^{C}), which consists of a globular α-helical-rich C-terminal domain, misfolds into a beta-plate-enriched conformer known as PrP^{Sc} (scrapie). Aggregates of PrP^{Sc} are pathognomonic for prion disease and signify the presence of prions. This will be discussed in more detail later in the thesis.

Prion diseases and the prion hypothesis

The prion diseases are characterized by a prolonged silent asymptomatic period, which can last up to 40 years in humans, during which no specific immune or inflammatory response can be recorded.

Table 2: The prion diseases

The prion diseases		
Disease	**Host**	**Mechanism of disease acquisition**
Kuru	Human	Infection through ritualistic cannibalism (eradicated)
Iatrogen CJD	Human	Medical and/or surgical treatment
Variant CJD	Human	Foodborne infection with BSE prions
Familial CJD	Human	Mutations in *PRNP*
Sporadic CJD	Human	Mutations or spontaneous conversion of PrP^{C} into PrP^{Sc}
FFI	Human	Mutations in *PRNP*
GSS	Human	Mutation in *PRNP*
SFI	Human	Mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}
Classical scrapie	Sheep and goats	Infection in genetically susceptible sheep and goats
Atypical scrapie	Sheep and goats	Probably sporadic prion disease in sheep and goats
Classical BSE	Cattle	Infection with prion-contaminated material
Atypical BSE **BSE-H** **BSE-L**	Cattle	Probably sporadic prion disease in cattle
TME	Mink	Infection with prions of unknown source
CWD	Cervids	Infectious
FSE	Cat	Foodborne infection with BSE prions

FFI: Fatal familial insomnia
GSS: Gerstmann-Sträussler-Schenker
SFI: Sporadic fatal insomnia
BSE: Bovine spongiform encephalopathy
BSE-H: High, based on the differences in PrP^{Sc} molecular masses identified after Western blot analysis.
BSE-L: Low, based on the differences in PrP^{Sc} molecular masses identified after Western blot analysis.
TME: Transmissible mink encephalopathy
CWD: Chronic wasting disease
FSE: Feline spongiform encephalopathy

In the central nervous system (CNS), neurodegeneration with neuronal loss, vacuolization, reactive gliosis (inflammation), and accumulation of misfolded PrP^{Sc} aggregates are observed (Masters and Richardson 1978, Williams, Lucassen et al. 1997). Our current understanding of prions and prion diseases result from a fascinating series of scientific explorations spanning most of the twentieth century, involving single case reports, prolonged transmission experiments, ritual cannibalism, rodent disease models, biochemical and structural studies, a large scale bovine epidemic, transgenics, two Nobel Prizes, and much more. Below, I will recapitulate some of the fundamental discoveries and turning points, which have led, ultimately, to the studies reported in this thesis.

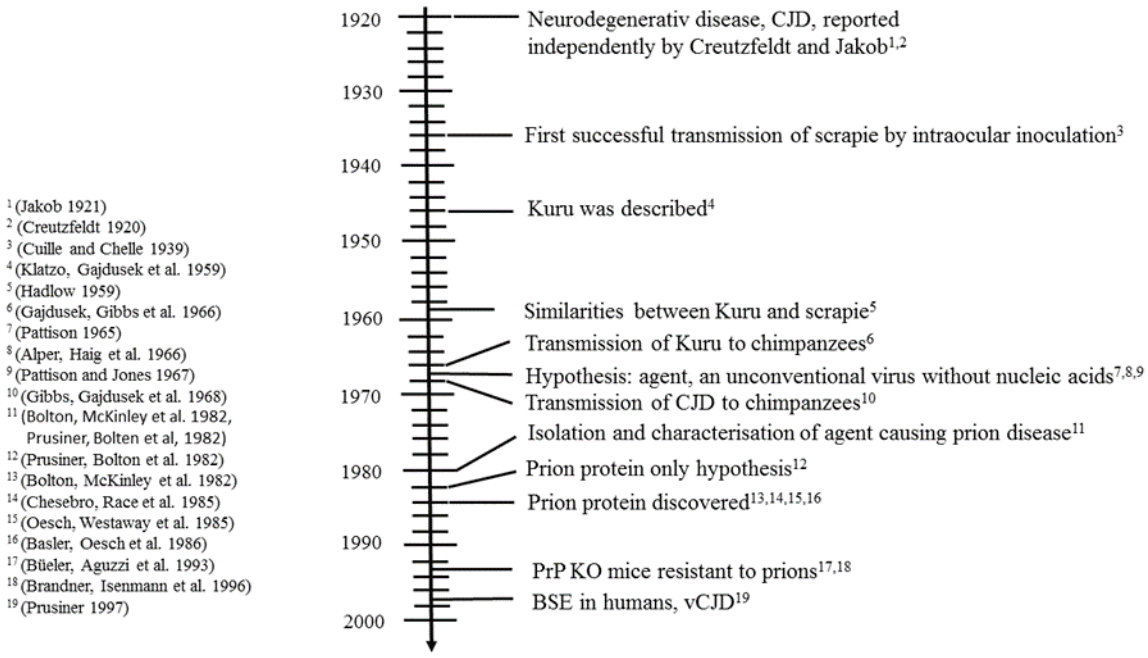

Figure 1: Timeline showing some of the major discoveries in the prion field
(Ref 19: (Prusiner 1997))

In the early 1920s, a new form of neurodegenerative disease was reported independently as case reports by Hans Gerhard Creutzfeldt (Creutzfeldt 1920) and Alfons Maria Jakob (Jakob 1921). When revising his major textbook in neurohistology in 1922, the German neuropathologist Walther Spielmeyer coined the terms Creutzfeldt-Jakob disease (CJD) and "status spongiosus" to characterize the histopathological observations.

Farmers and veterinarians in Europe had known scrapie in sheep for more than 250 years. In 1936, the first successful transmission of scrapie by intraocular inoculation of infected material was reported in a healthy sheep after a long incubation period (Cuille and Chelle 1939). This was the first evidence that this disease was transmissible, and the search for the infectious agent began. Due to the prolonged incubation period, the causative agent was believed to be a so-called slow virus or an unconventional virus (Sigurðsson 1954, Gajdusek 1977, Gajdusek 1985).

In 1959, Igor Klatzo recognized that the neuropathology of Kuru resembled that of CJD (Klatzo, Gajdusek et al. 1959), and, at the same time, veterinary pathologist William Hadlow made some important observations about Kuru. He realized that the histopathological characteristics of Kuru were strikingly similar to those seen in sheep scrapie (Hadlow 1959), which by then was established as a transmissible condition with a long incubation period.

This fact inspired Carleton Gajdusek and Michael Alpers, both working with Kuru in Papua New Guinea, to pursue further transmission experiments, and in 1966 they succeeded in transmitting Kuru to chimpanzees by intracerebral inoculation (Gajdusek, Gibbs et al. 1966). Prior to this, an outbreak of spongiform encephalopathy in farmed mink suggested that heterologous transmission of these diseases could occur (Hartsough and Burger 1965).

Two years later, in 1968, CJD was successfully transmitted to chimpanzees by intracerebral inoculation (Gibbs, Gajdusek et al. 1968).

Studies of the physiochemical features and physical robustness of the causative agent, revealed interesting and unusual traits. Most striking was the almost complete resistance to procedures such as ultraviolet radiation, which inactivates nucleic acids, but sensitivity towards procedures that modify or denature proteins (Alper, Haig et al. 1966, Gibbs, Gajdusek et al. 1978). This led to the hypothesis that the agent could be an unconventional virus without nucleic acids (Pattison 1965, Pattison and Jones 1967).

In 1982, Stanley B. Prusiner reported the isolation and characterization of the major component and the causative agent of prion disease after extensive studies of experimental prion disease in Syrian hamsters (Sha) (Bolton, McKinley et al. 1982, Prusiner, Bolton et al. 1982). The infective agent was named prion, a word derived from **pro**teinaceous **in**fectious particle (Prusiner, Bolton et al. 1982). The proteinase-resistant fragment of the scrapie isoform of the prion protein was a polypeptide of 27-

30 kDa (Prusiner, Groth et al. 1984). This proteinase-resistant protein core was found in the brain of animals and humans with prion disease (Bendheim, Barry et al. 1984), and appeared to be derived from a larger protein, called PrP^{Sc}. For the first time, scientists had a molecular marker that appeared to be specific for prion disease. Since the prion diseases had been linked to slow-acting viruses for many years (Sigurðsson 1954, Gajdusek 1977, Gajdusek 1985), it was assumed that PrP^{Sc} could be a viral protein. Thus, it was a surprise when PrP^{Sc} was identified as an isoform of a host-encoded cellular prion protein, PrP^{C} (Pan, Baldwin et al. 1993, Prusiner 1996). PrP^{C} is relatively rich in α-helical secondary structures and soluble in non-ionic detergents and readily degradable by proteases, while PrP^{Sc}, which is enriched with β-sheet secondary structures, is insoluble, and is partially resistant to digestion with proteinase K (Oesch, Westaway et al. 1985, Prusiner 1998). PrP^{C} and PrP^{Sc} differ only in their three-dimensional conformation and biochemical properties (Chesebro, Race et al. 1985, Oesch, Westaway et al. 1985, Basler, Oesch et al. 1986, Prusiner, Scott et al. 1998).

The pivotal role of PrP^{C} in prion disease has been demonstrated both epidemiologically and in several experimental ways. Most significant was the development of mice with ablation of the gene encoding PrP (*Prnp*) (PrP-KO) that proved to be completely resistant to prion infection (Bueler and Fischer 1992, Büeler, Aguzzi et al. 1993, Prusiner, Groth et al. 1993). Furthermore, it was shown that neuronal tissue derived from wild-type mice replicated prions after being grafted into the CNS of PrP-KO mice; however, without harming the surrounding PrP-negative brain tissue (Brandner, Isenmann et al. 1996). This demonstrated that neuronal expression of PrP^{C} is a prerequisite for prion replication and pathogenesis and that extracellular exposure to misfolded PrP^{Sc} is not toxic to PrP^{C}-negative neurons. Other aspects of the cell biology of PrP^{C} are also important, as shown in transgenic mice expressing secretory PrP^{C}, not membrane anchored, that did not succumb to disease nor generated prion infectivity, despite large amounts of extracellular PrP aggregates (Chesebro, Trifilo et al. 2005). Epidemiological and genetic analyses of human and sheep prion disease have also demonstrated that PrP^{C} is of fundamental importance for prion disease development. All known genetic forms of prion disease have been directly linked to specific mutations in the *PRNP* gene (Collinge 2001). In sheep, and several other species, polymorphisms in *PRNP* strongly influence prion-disease susceptibility.

Cellular prion protein

The mature prion protein (Figure 1) is a 210-residue (human numbering) cell surface glycosylphosphatidylinositol (GPI)-anchored protein (Stahl, Borchelt et al. 1987), encoded by the *PRNP* gene (Chesebro, Race et al. 1985, Oesch, Westaway et al. 1985, Basler, Oesch et al. 1986).

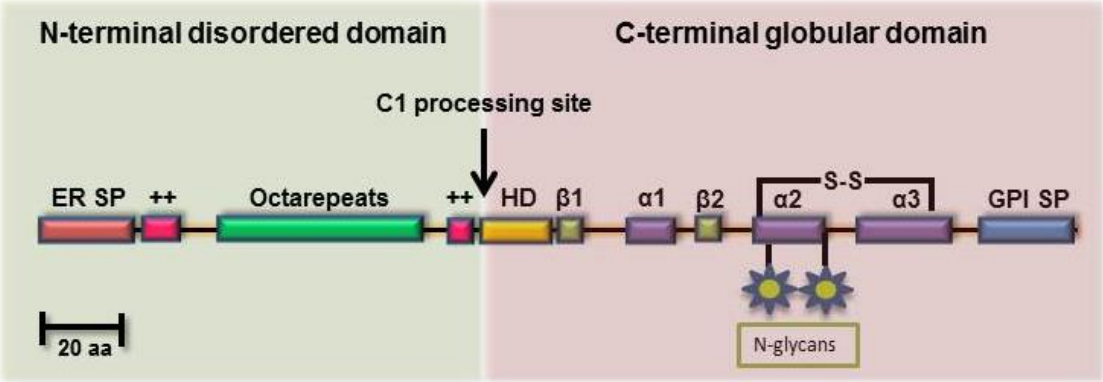

Figure 2: Structure of the cellular prion protein

Schematic illustration of the cellular prion protein with the N-terminal and C-terminal domain. The N-terminal domain is a disordered region of variable length, containing repeated sequences (green). The C-terminal domain consists of three helices (α1, α2, α3) and a β-sheet formed by two β-strands (β1, β2). C1 processing site illustrate the proteolytic cleavage site, known as α-cleavage (arrow). Adopted from (Bakkebø, Mouillet-Richard et al. 2015).

It is co-translationally translocated into the endoplasmic reticulum (ER) and transits through the secretory route to the cell surface (Harris 1999). In the ER, the N-terminal signal sequence (residues 1–22) (Figure 2) and the C-terminal hydrophobic segment (231–253) are cleaved. The GPI-anchor is attached to the C-terminus of the protein and two asparagine (N)-linked oligosaccharides are added to residues Asn 181 and Asn 197 (Stahl, Borchelt et al. 1987, Haraguchi, Fisher et al. 1989, Rudd, Merry et al. 2002). The oligosaccharides, N-glycans, are further modified in the Golgi (Stahl, Baldwin et al. 1992). Interestingly, PrP^{C} normally appears in three major glycoforms, namely di-glycosylated form, which is dominating, mono-glycosylated form, and a minor fraction without glycans. The function of the N-glycans could be to provide increased stability of the protein, as well as to regulate the sub-cellular sorting and

trafficking of PrP^C. The GPI-anchor is necessary for tethering PrP^C to the plasma membrane and for the localization of PrP^C to cholesterol and sphingolipid-enriched membrane sub-domains, known as membrane rafts. It has been demonstrated that GPI-anchoring of PrP^C is crucial for prion replication and pathology (Taraboulos, Scott et al. 1995, Brandner, Isenmann et al. 1996, Mallucci, Dickinson et al. 2003, Chesebro, Trifilo et al. 2005).

It should be noted that several lines of evidence have shown that PrP^C displays pleiotropic sub-cellular localizations, including nucleo-cytoplasmic localization (Gu, Hinnerwisch et al. 2003, Crozet, Vézilier et al. 2006). The physiological roles of cytoplasmic or nuclear PrP^C fragments generated by proteolytic cleavage have not been elucidated, nor the precise mechanisms or the cellular conditions that govern the generation of these protein species. At least three different mechanisms have been proposed to explain the generation of cytoplasmic PrP^C, two of which are related to stressful conditions in the ER, blocking PrP^C from entry (Kang, Rane et al. 2006) or retro-translocation of PrP^C from the ER to the cytoplasm (Yedidia, Horonchik et al. 2001, Ma and Lindquist 2002) as part of an unfolded protein stressresponse. The third mechanism involves alternative translation initiation and a phenomenon known as leaky ribosomal scanning, which results in use of an alternative, downstream start codon. This leads, in the case of PrP^C, to a shortening of the ER targeting signal sequence and cytoplasmic localization instead of ER import (Juanes, Elvira et al. 2009, Lund, Olsen et al. 2009). It has been demonstrated that two clusters of positively charged residues in PrP^C (See Figure 2) can function as nuclear localization signals (NLS) (Gu, Hinnerwisch et al. 2003). Several reports have shown that PrP^C can bind to nucleic acids (Silva, Lima et al. 2008) and bend DNA (Bera, Roche et al. 2007) and associate with chromatid structures, such as Histone 3 (Cai, Xie et al. 2013). Thus, it cannot be ruled out that a sub-species of PrP^C, possibly constituting a minority of total cell PrP^C, acts in a nucleo-cytoplasmic compartment of the cell. In 2014, a direct role for PrP^C in DNA repair has also been proposed, involving translocation of full length PrP^C with glycan moieties into the cell nucleus (Bravard, Auvré et al. 2014). I will return to this in more detail in the discussion of the thesis.

Studies of brain-derived PrP^C by two-dimensional immunoblots have revealed a significant heterogeneity in PrP^C species, signifying a complex cellular biology with several proteolytic processing events (Pan, Li et al. 2002). Before this, it had been

shown that PrP^C undergoes proteolytic processing, particularly prevalent at a site just N-terminal to the central hydrophobic domain of PrP^C, as shown in Figure 2 (Pan, Stahl et al. 1992, Harris, Huber et al. 1993, Shyng, Huber et al. 1993, Chen, Teplow et al. 1995). This cleavage site is known as the α-cleavage site, whereas another site, some residues closer to the N-terminus, is known as the β-cleavage site (Mangé, Béranger et al. 2004), and cleavage at this site appears to be initiated by reactive oxygen species (ROS). The conserved α-cleavage can occur during transit through the Golgi network, and apparently independent of GPI-anchoring of PrP^C (Tveit, Lund et al. 2005, Walmsley, Watt et al. 2009). Furthermore, full length PrP^C has been shown to be released from the plasma membrane by one or more cleavages near or within the GPI-anchor in a shedding process, possibly catalysed by ADAM10 (Altmeppen, Prox et al. 2011). The α-cleavage occurs within the highly conserved hydrophobic region (residues 106–126), which has neurotoxic properties (Chiesa and Harris 2001) and it has been proposed that inhibition of the α-cleavage during prion disease might contribute to PrP^C-derived toxicity (McDonald and Millhauser 2014). PrP^C is processed into two fragments; PrP-N1 and PrP-C1. The N-terminal fragment is secreted, whereas the C-terminal fragment remains attached to the cell membrane. The ROS-activated β-cleavage generates the fragments PrP-N2 and PrP-C2 (Chen, Teplow et al. 1995, McDonald and Millhauser 2014). This might be a mechanism by which PrP^C protects cells against oxidative stress. PrP^C lacking the octapeptide repeat region fails to undergo ROS-mediated β-cleavage. This is associated with two inherited forms of prion disease in humans (Watt, Taylor et al. 2005).

The normal function of PrP^C still remains elusive. However, PrP^C may play a role in cell survival, signal transduction, protection against oxidative stress (Brown, Nicholas et al. 2002), apoptosis (Bounhar, Zhang et al. 2001, Roucou, Gains et al. 2004), and immunological quiescence (Bakkebø, Mouillet-Richard et al. 2015). It has been shown that PrP^C is upregulated after focal cerebral ischemia (Weise, Crome et al. 2004) and that overexpression reduces the extent of neuronal loss after ischemic insult, suggesting that PrP^C might have a neuroprotective capacity (Shyu, Lin et al. 2005). It has also been proposed that PrP^C may regulate neuronal precursor proliferation during developmental and adult mammalian neurogenesis (Steele, Emsley et al. 2006). *Prnp* KO mice are healthy throughout their lifespan, with normal development. However, as mentioned above, they are resistant to scrapie infection

(Büeler, Aguzzi et al. 1993). A large number of studies involving *Prnp* KO mice have been carried out, which has resulted in a correspondingly large catalogue of putative roles for PrP^C function (reviewed in (Onodera, Sakudo et al. 2014)). Likewise, a number of studies have compared cell lines with and without expression of *Prnp* to gain insights into PrP^C normal function (Onodera, Sakudo et al. 2014).
As mentioned, PrP^C has metal-binding properties. More precisely, the octameric repeat region in the N-terminal part of the protein can bind up to four copper atoms *in vivo*, and it has been suggested that PrP^C thus might express Cu/Zn superoxide dismutase (SOD) activity by influencing copper incorporation into SOD (Brown, Clive et al. 2001). Expression of PrP^C with copper bound appears to enhance cellular resistance to oxidative stress (Brown, Wong et al. 1999, Brown, Clive et al. 2001). Deletion or deficiency of this part of the protein abolishes the PrP^C-dependent SOD activity (Brown, Wong et al. 1999) or reduces the activity of cytochrome C oxidase. This might result in mitochondrial dysfunction, with a concomitant increase in ROS generation. This can trigger mitochondria-mediated apoptotic neurodegeneration (Rossi, Lombardo et al. 2004). Impaired Cu/Zn SOD is believed to be one of the mechanisms involved in neurodegenerative diseases (Bruijn, Miller et al. 2004).

Similarities between Alzheimer's and prion disease

When looking at similarities between AD and prion diseases, it is interesting to examine the proteins APP and PrP closely. Both proteins are synthesized into the secretory route and destined for the plasma membrane. APP is an integral membrane protein, whereas PrP^C is tethered to the membrane with a glycolipid anchor. During transit through the secretory pathway, both proteins are subject to glycosylation and complex proteolytic processing. Both proteins also have metal-binding capacities, particularly for divalent cations, like Fe^{2+}, Cu^{2+} and Zn^{2+}.
It has been shown that deposits of Aβ directly contribute to ROS generation upon binding divalent cations, and, indirectly, by stimulation of mitochondrial ROS production and initiating an inflammatory response in microglial cells (Tuppo and Arias 2005). Interestingly, PrP^C has been reported to mediate Aβ toxicity, by functioning as a cell surface Aβ receptor and directly playing a role in AD pathology (Laurén, Gimbel et al. 2009, Zou, Xiao et al. 2011, Kudo, Lee et al. 2012, Larson, Sherman et al. 2012, You, Tsutsui et al. 2012). It has been demonstrated that PrP^C

can bind a diversity of beta-plate-enriched proteins (Laurén, Gimbel et al. 2009, Chen, Yadav et al. 2010, Moos, Calella et al. 2010), independent of prion replication, suggesting that PrP^{C} might act as a low-specificity receptor with the potential of mediating various pathologies in addition to prion disease (Kessels, Nguyen et al. 2010, Kudo, Lee et al. 2012).

Moreover, it has been reported that extracellular plaques in AD patients can also contain PrP^{Sc} (Esiri, Carter et al. 2000, Ferrer, Blanco et al. 2001), and, conversely, that plaques found in the human prion disease CJD can contain Aβ deposits (Miyazono, Kitamoto et al. 1992, Hainfellner, Wanschitz et al. 1998).

Transmissibility, previously thought to be a peculiarity of prion disease, was observed in a mouse model of AD, in which intraperitoneal injection of Aβ seeds accelerated Aβ deposits in recipient mice genetically engineered for amyloid plaque development (Kane, Lipinski et al. 2000, Meyer-Luehmann, Coomaraswamy et al. 2006, Eisele, Obermüller et al. 2010). However, administration of brain extract through an oral, intravenous, intraocular, or intranasal route did not lead to cerebral β-amyloidosis, in contrast to the experimental transmission of prion diseases (Eisele, Bolmont et al. 2009).

Experimental prion disease and prion strains

A characteristic of prion disease, unlike all other proteinopathies, is the transmissibility of the disease either naturally, such as in classical scrapie in sheep and CWD among cervids, or experimentally, in suitable hosts. Experimental transmission has been of tremendous importance in exploring these maladies. The rodent animal models, in particular, have enabled detailed characterization of disease development and progression, as well as investigation of the properties of the transmissible prions themselves. After several passages in a mouse model, with successive re-isolation of infectivity, a prion isolate will become mouse-adapted, meaning that infection of this line of mice with this particular prion isolate will generate a highly reproducible disease, in terms of incubation period and distribution of pathological lesions (Dickinson, Meikle et al. 1968, Fraser and Dickinson 1968). The mouse-adapted strain used in our studies is one such strain. The terms “prion strain” and “species barrier” stem from original observations of heterologous prion transmissions, after which a large reduction in incubation periods from first to second passage was

observed, suggesting that the prolonged incubation period for the first passage represents a barrier for transmission, whereas upon second and subsequent passages this barrier had been broken. When stabilized after some passages, a prion isolate will, as pointed out above, give rise to a very reproducible incubation period (Dickinson, Meikle et al. 1968), which is characteristic for the isolate. Moreover, the distribution of histopathological lesions (lesion profile), especially the degree of vacuolization of the brain parenchyma, would also be reproduced, and thus, be a further characteristic feature of that prion isolate (Fraser and Dickinson 1968). The existence of several scrapie isolates with strain properties as indicated above, and the intriguing observation of what seemed to be strain "mutations" in which an isolate could switch into another strain upon suitable passage, led to heated controversy in the prion field for many years. Could these observations of complex biological variation be explained for infectious agents devoid of nucleic acids, as dictated by the "protein-only" hypothesis? Today, it is commonly accepted that prion strains are derived from structural variation in aggregates of PrP^{Sc} (Hecker, Taraboulos et al. 1992, DeArmond, Yang et al. 1993) and that these structural features are inherited through the process of prion replication. The existence of multiple prion strains can also be explained by diverse prion protein glycosylation and conformation patterns (Collinge 2001).

In an infectious isolate, several structural entities might be present in various amounts and these might undergo a selection process upon replication in different hosts or experimental settings, thus allowing different strains to emerge (Halliez, Reine et al. 2014), reviewed in (Béringue, Vilotte et al. 2008).
Mouse-adapted scrapie with relatively short incubation periods, allows rapid and reproducible studies, recapitulating all major aspects of naturally occurring prion disease. This is a significant advantage compared with the mouse models for AD and PD, which only reproduce some aspects of the diseases.

The Rocky Mountain Laboratories mouse-adapted scrapie strain, RML.
Several mouse-adapted strains were derived from a pooled sample of brains from scrapie-infected sheep, known as SSBP/1. Passaging of SSBP/1 in mice resulted in the "22 family" of strains (Dickinson and Fraser 1979). Transmission of the same infectious material to goats resulted in isolation of two clinically distinct strains of

scrapie, namely the "scratching" and "drowsy" strains (Pattison and Millson 1961). The "drowsy" variant from goat, when passaged in mouse, resulted in the isolation of the mouse-adapted "Chandler" strain (Chandler 1961), from which the RML strain was subsequently developed upon serial passage.

Pathogenesis in prion disease

A direct interaction between the correctly folded PrP^{C} and the infectious misfolded protein, PrP^{Sc}, is required for the conformational transition to occur (Harris 1999). It is believed that PrP^{Sc} aggregates are capable of seeding a self-perpetuating reaction. Despite decades of research, the precise mechanism and cellular site where the conversion occurs remain unknown.

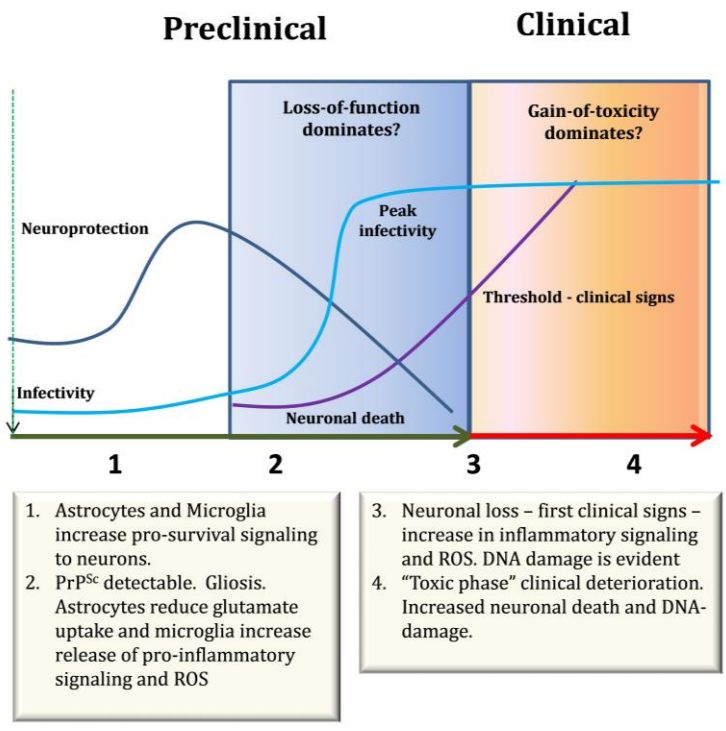

Figure 3: Pathogenesis in prion disease

Schematic representation of prion disease pathogenesis, highlighting the long pre-clinical stage that precedes the short, clinical, so-called toxic phase of disease. Dynamics of neuroprotection, infectivity build-up and neuronal death is illustrated by curves and split arbitrarily into four stages (1-4). The figure draws inspiration from several papers on the subject of prion pathogenesis, such as (Soto and

Satani 2011, Aguzzi, Barres et al. 2013, Sandberg, Al-Doujaily et al. 2014, Diack, Alibhai et al. 2016). The figure indicates, tentatively, that loss-of-PrP-function dominates in the pre-clinical phase whereas gain-of-toxicity is more prevalent in the toxic phase.

When the conversion takes place within the CNS, a neurotoxic signal is at some stage triggered, plausibly through PrP^C itself. In the early pre-clinical stage of brain prion disease, astrocytes and microglia respond by increasing pro-survival signals to the neurons (Aguzzi, Barres et al. 2013).

Microglia, the resident macrophages of the brain and spinal cord, are crucial as a first line of immunological defence. In the normal brain, microglia were considered "resting", but two-photon microscopy has shown that microglia constantly scan the brain environment in an effort to maintain homeostasis (Nimmerjahn, Kirchhoff et al. 2005). In the case of brain damage or injury, microglia can become activated and undergo morphological, as well as functional, transformations (Aguzzi, Barres et al. 2013). They can remove damaged cells and cellular debris, as well as dysfunctional synapses, through phagocytosis (Kettenmann, Kirchhoff et al. 2013). Another prominent function of microglia, which is evident in prion disease, is release of pro-inflammatory mediators (Burwinkel, Riemer et al. 2004, Rock, Gekker et al. 2004), such as cytokines (Boche, Perry et al. 2013) and ROS (Tuppo and Arias 2005). Cytokine production is essential for the activation of microglia into what has been termed a classically activated "M1" state (Martinez 2011, Aguzzi, Barres et al. 2013). Released cytokines can recruit mononuclear phagocytes to the developing brain (Rezaie and Male 1999), during CNS inflammation (Hesselgesser and Horuk 1999, Mennicken, Maki et al. 1999) and in neurodegenerative disorders, including AD (Xia and Hyman 1999). Microglia, as well as other CNS cells, such as astrocytes, can also produce tumor necrosis factor alpha (TNF-α) (Cherry, Olschowka et al. 2014), which induces neurogenesis, with proliferation of neural stem cells, and growth factors (Butovsky, Ziv et al. 2006, Hanisch and Kettenmann 2007, Nakajima, Tohyama et al. 2007). This response is protective and will be downregulated once the pathogen is eliminated or homeostasis is restored. A sustained activation during, for instance, chronic inflammation can lead to tissue destruction. As illustrated in Figure 3, the neuroprotective processes are gradually reduced as the prion replication increases. The astrocytes reduce glutamate uptake and the microglia increase the release of cytokines and ROS. At the same time neuronal function is reduced, and, finally,

degeneration of axon terminals and vacuolization occur before onset of clinical disease (Jeffrey, Goodsir et al. 1997, Jeffrey, Halliday et al. 2000).

Progressive dysfunction and death of neurons are often associated with aggregates of misfolded proteins (Soto and Estrada 2008), and increased oxidative stress is believed to be of major importance (Wang and Michaelis 2010), reviewed in (Finkel and Holbrook 2000). Chronically increased levels of ROS will induce irreversible modifications of proteins, lipids, and nucleic acids, and lead to elevated levels of intracellular calcium, and gradual decline in mitochondrial function (Behl, Davis et al. 1994), which, in turn, will further increase ROS generation. ROS will induce damage to nuclear and mitochondrial DNA. As previously mentioned, the cellular functions of PrP^{C} are not known in detail, but it has been demonstrated in a variety of experimental settings that the protein might contribute to cell-protective signalling during stressful conditions (Brown, Schulz-Schaeffer et al. 1997, Kuwahara, Takeuchi et al. 1999, Watt, Routledge et al. 2007). Therefore, it has been assumed that at least some of the neuronal deterioration seen during prion disease might stem from loss of PrP^{C}'s function. In support of this, transgenic expression of mutated, dysfunctional PrPs have, in some cases, led to neurodegeneration in the absence of prion replication and accumulation of misfolded PrP (Resenberger, Winklhofer et al. 2011). For instance, expression of a deletion mutant of PrP, known as PrPΔHD, in which amino acids 105–125 have been deleted, leads to spontaneous neurodegenerative disease. This can be prevented by co-expression of normal PrP, presumably restoring normal function. However, this could also be interpreted slightly differently; namely, that PrPΔHD not only leads to loss of PrP function but initiates toxic signalling on its own, which can be blocked by normal PrP (Resenberger et al 2011). The lack of overt neurological symptoms in transgenic PrP KO animals and goats naturally devoid of PrP also apparently contradict the loss-of-function hypothesis, although hitherto unknown compensatory mechanisms might confound this. As illustrated in Figure 2, it is likely that during prion disease a combination of loss-of-function and gain-of-toxicity most likely occur. This is not only related to PrP and its misfolded conformers, but more generally expressed as neuroprotective versus neurotoxic signalling. It is a universal biological phenomenon that perturbation of homeostasis elicits a series of counteractive and compensatory measures aimed at restoring the balance. However, in the face of sustained stress, counteractive measures, such as DNA repair mechanisms, might be gradually overwhelmed, and the cellular ability to restore homeostasis is

lost. Vicious circles might develop, dominated by escalating pro-inflammatory and toxic signalling. As shown in Figure 3, we consider this to be a dominant feature during the clinical stages of prion disease in which a rapid neurological deterioration is observed. It is interesting to note that most of the pathological features that characterize prion diseases, such as gliosis, are already present at the onset of clinical disease. In our experimental model, we observe an increase in brain vacuolization and increase in DNA damage, both in the nucleus and particularly in mitochondria during the clinical stage. It is, without doubt, the escalating neuronal death that is the main driver of clinical symptoms during the final, so-called "toxic phase", of prion disease.

Centrifugal spread of prions after intracerebral inoculation and peripheral pathology

The natural route of prion infection is usually through extracerebral infection, but clinical disease occurs only after invasion of the CNS. As mentioned earlier, the prion protein is necessary for prion infection and disease. A lack of anchored PrP appears to affect the speed and efficiency of neuroinvasion (Klingeborn, Race et al. 2011). After intra-cerebral inoculation, prion propagation accrues first in the autonomic nerves, then accumulate in the lymphoid organs, before PrP^{Sc} is transported to the spleen in a centrifugal manner (Crozet, Lezmi et al. 2007).

The development of neurological disease after peripheral inoculation depends on prion expansion within cells of the lymphoreticular system. In this peripheral phase of the disease, lymphoid organs (Aguzzi, Montrasio et al. 2001), immune cells (Klein, Frigg et al. 1997, Klein, Frigg et al. 1998), and peripheral nerves (Race, Priola et al. 1995, Glatzel, Heppner et al. 2001) are involved. It is likely that prions transfer between lymphoid organs by circulating mononuclear phagocytes (Wathne and Mabbott 2012). In lymphoid organs, the agent appears to accumulate in follicular dendritic cells (FDC) (Klingeborn, Race et al. 2011) and within the extracellular spaces surrounding the dendrites (Jeffrey, McGovern et al. 2000) of lymphoid tissue such as the spleen, lymph nodes, tonsils, the appendix, and gut Peyer's patches, before spreading to the nervous system. The early accumulation of PrP^{Sc} in lymphoid tissues is essential for efficient neuroinvasion. An absence of lymphoid tissues like Peyer's patches (Prinz, Huber et al. 2003) or spleen (Crozet, Lezmi et al. 2007) impairs neuroinvasion.

It has been observed by others and also seen in our studies that after intracerebral inoculation, prions rapidly reach the general circulation and a number of peripheral organs, most notably in the lymphoreticular system (Kimberlin and Walker 1979). Most notably, prion propagation rapidly ensues in the spleen after intracerebral inoculation, due to spillover from the brain (Langevin, Andréoletti et al. 2011). It has long been known that the spleen is involved early in the prion pathogenesis. In our studies, summarized in paper III, we have focused particularly on the spleen even if the main pathology occurs in the CNS (Kimberlin and Walker 1988). Since it was previously reported that *Neil2*$^{-/-}$-mice are hyper-responsive towards certain pro-inflammatory agents, we were particularly interested in studying the progression of prion disease in the spleen, in terms of gene expression, DNA damage and morphology. Due to very early peak levels of infectivity in the spleen just 2-3 weeks after ic inoculation, (Rubenstein, Merz et al. 1991, Race, Ernst et al. 1992), which is well before peak of infectivity in the brain occurs, it was of major interest to investigate the function, morphology and pathology of the spleen.

ROS-mediated DNA damage

The brain constitutes 2 % of the bodyweight, but consumes about 20 % of the inhaled oxygen. Moreover, it possesses low levels of antioxidant enzymes, which renders the brain susceptible to oxidative stress (Halliwell 1992). Proteins, lipids, and nucleic acids can be harmed by oxidation. Such oxidative damage is thought to be important in the pathogenic processes leading to slowly developing diseases, such as cardiovascular diseases, type 2 diabetes, and several neurodegenerative diseases, e.g. AD (Christen 2000). In AD, it is believed that an age-related accumulation of ROS results in damage to major components of the cell, e.g. nuclear DNA (nDNA), mitochondrial DNA (mtDNA) and membranes, in addition to cytoplasmic proteins.

Neuronal cells are terminally differentiated, post-mitotic cells with a high rate of oxygen consumption. The neurons appear to be highly sensitive to attacks by ROS, due to the possession of a low content of glutathione (Cooper and Meister 1997), an important natural antioxidant. The neuronal cells are dependent on an efficient repair pathway, because of their limited cellular regenerative capacity compared with other terminally differentiated cells (Vierck, O'Reilly et al. 2000, Nouspikel and Hanawalt

2002), but also because of the high transcriptional demand. Accumulation of ROS-mediated DNA damage has been observed in multiple neurodegenerative disorders, including AD (Hartman 1995). In AD, aberrant metal homeostasis may contribute to the formation of ROS and toxic Aβ oligomers, facilitating the formation of amyloid plaques (Maynard, Bush et al. 2005), and increased lipid peroxidation precedes Aβ plaque formation in an animal model of AD (Praticò, Uryu et al. 2001). The studies presented in this thesis specifically address the question of putative roles of the base excision repair (BER) pathway DNA repair in the face of prion pathology, by inducing experimental prion disease in novel lines of transgenic mice that are compromised in BER pathway DNA repair.

DNA repair

In the 1970s, many scientists thought that the DNA molecule was stable, but Professor Tomas Lindahl demonstrated that it decays. This later led him to discover the mechanisms of base excision repair (BER). In 2015, Tomas Lindahl, together with Paul Modrich and Aziz Sancar, received the Nobel Prize in Chemistry for discoveries on DNA repair.

DNA is constantly exposed to endogenous cellular metabolic by-products, and exogenous environmental factors, such as genotoxic chemicals, viruses, and UV radiation, all of which can lead to DNA damage and destabilize the structure (Hakem 2008). In addition, spontaneous damage occurs at a high rate due to the inherent chemical instability of DNA (Lindahl 1993). The cellular mechanisms to protect against metabolic processes consist of an elaborate antioxidant defence system featuring several enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and peroxyredoxins, and low-molecular-mass scavengers, such as glutathione (Finkel and Holbrook 2000).

A single human cell sustains more than 10,000 DNA lesions every day from endogenous sources (Wallace, Murphy et al. 2012). ROS, such as superoxide radical anions, hydrogen peroxide, or hydroxyl radicals, are formed as by-products of oxygen metabolism and can react with DNA to give rise to more than 100 different modifications in DNA. Normally, ROS is also involved in physiological processes, including intracellular signalling (Ray, Huang et al. 2012), proliferation and

differentiation of embryonic stem cells (Nitti, Furfaro et al. 2010, Le Belle, Orozco et al. 2011), and apoptosis. An imbalance between oxidants and antioxidants, results in increased levels of cellular damage (David, O'shea et al. 2007).

Mitochondrial DNA (mtDNA) is more exposed to ROS-induced DNA damage than nuclear DNA (nDNA), probably due to the proximity to ROS production in the mitochondria and different organization of the DNA (Yakes and Van Houten 1997). The oxidative attack will result in DNA strand breaks, base modifications, DNA-protein cross linking, and apurine/apyrimidine sites (AP sites) in the DNA (Bjelland and Seeberg 2003). These lesions are normally repaired by the BER pathway. Permanent defects in DNA repair is associated with severe consequences, including impaired growth, rapid aging, neurodegeneration, embryonic lethality (Hoeijmakers 2001), and cancer predisposition in rapidly dividing cell types. In post-mitotic, non-dividing cells, a defect in DNA repair will lead to a decline in function and potentially cause degeneration and cell death.

DNA damage can be removed by six different pathways: BER, nucleotide excision repair, direct DNA damage reversal, mismatch repair, and two pathways, which are responsible for the repair of double-strand breaks (DSB); homologous recombination and non-homologous end joining. In the scope of this thesis the focus is on the BER pathway.

DNA repair in the CNS and neurons

The CNS consists of the brain and the spinal cord with many different cell types, including neurons and glial cells. These neurons are terminally differentiated, which means that they only repair their transcribed genes and not the rest of their genome. Nouspikel et al found that both DNA strands of the active genes are repaired efficiently (Nouspikel and Hanawalt 2002). This mechanism may be required when global genomic repair has been shut off, because the non-transcribed strand is needed as a template to repair the transcribed strand. If neurons accumulate lesions in non-transcribed strands over an extended period, this would be likely to induce mutations when the damaged template was used for repair. A deficiency in this differentiation-associated repair would be expected to result in age-related inactivation of genes,

leading to progressive metabolic dysfunction and ultimately to premature neuron "aging" and cell death, causing early dementia (Nouspikel and Hanawalt 2002).

Base excision repair pathway

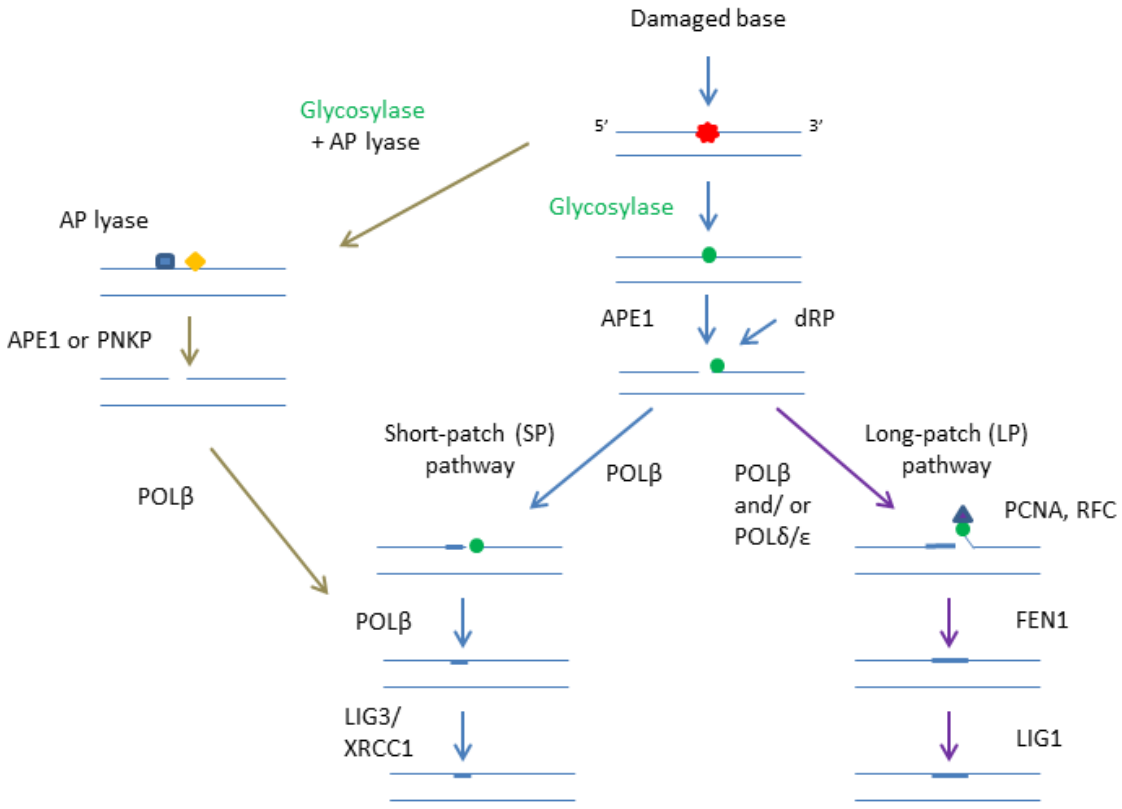

Figure 4: The BER pathway.

Repair is initiated by mono- or bifunctional DNA glycosylases, excising the base lesions from the DNA backbone. The predominant short-patch BER pathway is indicated by blue arrows. The enzymes that catalyse each step are indicated. The BER pathway, initiated by the bifunctional DNA glycosylase/ AP lyase is marked with brown arrows. In long-patch pathway, marked in purple, POLδ/ε and associated factors introduce two to six nucleotides past the abasic site. The resulting overhang is excised by FEN1 endonuclease and the nick is sealed by DNA ligase 1. ● Apurinic/apyrimidinic site. ■ AP, Phosphate (P) or unsaturated aldehyde (UA), ◆ Hydroxylgroup (OH). dRp 5'-deoxyribose phosphate group, Proliferating cell nuclear antigen (PCNA), Replication factor C (RFC), Flap nuclease 1(FEN1), Ligase (LIG), X-ray repair cross-complementing protein 1 (XRCC1). Modified from (Maynard, Schurman et al. 2009).

BER is the major pathway that is responsible for removing oxidative DNA base lesions from both nDNA and mtDNA and for restoring the integrity of the genome. This pathway is initiated by DNA glycosylases, a class of enzymes that recognize a specific set of modified bases due to alkylation, oxidation, or deamination (Krokan and Bjørås 2013). DNA glycosylases are small proteins, 30-50 kDa, which cleave the N-C1' glycosylic bond between the base and deoxyribose, thus releasing the damaged base and leaving an apurinic/apyrimidinic site (AP site) (Krokan, Standal et al. 1997, Schärer 2003, Barnes and Lindahl 2004, Jacobs and Schär 2012).

DNA glycosylases can be monofunctional (Figure 4; blue arrows), which means that they only have glycosylase activity, thus producing an AP site. This site is further processed by an AP endonuclease 1, APE1 (Wilson Iii and Barsky 2001, Demple and Sung 2005), which nicks the DNA backbone 5' to the AP site. DNA polymerase β (POL β) removes the resulting 5' deoxyribose phosphate (dRP), producing a single nucleotide gap. The remaining nick is sealed by DNA ligase.
Glycosylases with bifunctional activity (Figure 4; brown arrows) have a β-lyase activity or a β, δ-lyase activity that cleaves the DNA strand in addition to the glycosylase activity. The β-eliminations result in a 3'phosphor α,β unsaturated aldehyde (PUA) end, while the β, δ-elimination results in a 3' phosphate (P) end, leaving ends that require tailoring before BER can proceed (Svilar, Goellner et al. 2011, Jacobs and Schär 2012). There are at least 11 different mammalian DNA glycosylases, including mammalian NEIL family (Bandaru, Sunkara et al. 2002, Hazra, Izumi et al. 2002, Morland, Rolseth et al. 2002), where the three members are homologous to the *Escherichia coli* (*E. coli)* Formamidopyrimidine DNA glycosylase (Fpg) and Endonuclease VIII (Nei) proteins (Wallace, Bandaru et al. 2003, Zharkov, Shoham et al. 2003).
These glycosylases, together with NTHL1, are bifunctional and specialized for removal of oxidized bases.
In short-patch BER (SP-BER) (Figure 4), also known as single nucleotide BER, Pol β inserts the missing base. The nick is sealed by DNA ligase III (LIG III) complexed to X-ray repair cross-complementing protein 1 (XRRCC1) (Ellenberger and Tomkinson 2008). However, recent evidence suggests that DNA ligase I (LIG I) may be the major nuclear DNA ligase, both in short-patch and long-patch BER, whereas DNA ligase III is essential in mitochondria (Gao, Katyal et al. 2011, Simsek, Furda et al. 2011).

Short-patch BER is equally efficient in proliferating and nonproliferating cells.

Long-patch BER (LP-BER) (Figure 4) occurs mainly in proliferating cells, where a polymerase (β, δ, ε) fills in the gap and keeps synthesizing DNA while displacing the DNA downstream of the initial damage site, creating a flap of DNA. The flap structure is subsequently incised by flap endonuclease 1 (FEN1) and the nick is sealed by LIG I.
The choice of pathway depends on the initiating glycosylase, the cell type, and the availability of BER factors. For oxidative base damage and SSB, the LP-BER is proposed to be highly relevant (Fortini and Dogliotti 2007).

BER is not only involved in DNA repair, but seems to be involved in the maintenance of epigenetic stability as well (Cortázar, Kunz et al. 2011).

Table 3: DNA glycosylases, their function and the mutant phenotypes

Enzyme	Mono/ bifunctional	Type of base lesion	Mouse knockout phenotype:
OGG1	Bifunctional	Oxidative base damage	ko: viable, accumulation of 8-oxoG,
8-oxoG DNA glycosylase 1			elevated mutation frequency (G→T)
MYH MutY	Monofunctional	Oxidative base damage	ko: viable, see OGG1
homolog DNA glycosylase			
NEIL2	Bifunctional	Oxidized	Increased inflammatory responsiveness age-dependent accumulation of oxidative DNA damage
Endonuclease VIII-like		ring-fragmented or	
glycosylase 2		saturated pyrimidines	
NEIL3	Bifunctional	Oxidized	Memory deficit Compromised neurogenesis
Endonuclease VIII-like		ring-fragmented or	
glycosylase 3		saturated pyrimidines	

Modified from (Jacobs and Schär 2012)

Mouse models for deciphering BER pathway enzymes

Genetically engineered mice, deficient in components of the BER pathway, have been used to study the consequences of DNA damage, accumulation, mutations, and carcinogenesis in different cells (Larsen, Meza et al. 2007). The phenotypes observed in mice with genetic ablation of BER enzymes vary and do not necessarily present an immediate or obvious phenotype, whereas intermediate and late stage BER gene targeting may result in embryonic lethality (Xanthoudakis, Smeyne et al. 1996, Larsen, Gran et al. 2003, Izumi, Brown et al. 2005, Puebla-Osorio, Lacey et al. 2006).

The severity of DNA damage depends on the cell properties. Replicating cells are more likely to acquire mutations, which may lead to malignant disease, whereas terminally differentiated cells accumulate DNA damage, which ultimately leads to cell death and degenerative disease.

Ogg1/ Mutyh mice

Experimental prion disease in mice is a valuable and efficient tool for studying the roles of ROS-mediated DNA damage and specific DNA repair enzymes in the pathogenesis of slowly developing neurodegenerative diseases. One challenge, particularly relevant in paper I in this thesis, is that some lines of DNA repair-compromised mice are prone to cancer. This is true for the line of mice with double knockout of *Ogg1* and *Mutyh.* However, as demonstrated in that paper, the relative rapidity of RML prion disease allowed analysis of prion disease to be performed before the onset of cancer at around 6-8 months (Xie, Yang et al. 2004).

In contrast to most other animal models of neurodegeneration, prion-infected mice undergo a highly stereotypic course of disease with a well-defined incubation and survival time, but also with clear endpoints and reliable biomarkers.

Mice with ablation of Neil genes

As mentioned, the first step of the BER pathway consists of DNA glycosylases recognizing modified bases. The three mammalian DNA-glycosylases, NEIL1, NEIL2, and NEIL3, were discovered by several research groups 15 years ago

(Bandaru, Sunkara et al. 2002, Hazra, Izumi et al. 2002, Morland, Rolseth et al. 2002, Takao, Kanno et al. 2002). These DNA glycosylases show sequence similarity to *E. coli* Endonuclease VIII (Nei), and are therefore named Nei-like (NEIL).

NEIL1 and NEIL2 are ubiquitous in humans and mice, but the level of expression differs between organs (Hazra, Izumi et al. 2002, Hazra, Kow et al. 2002, Morland, Rolseth et al. 2002). The expression of mouse *Neil3* is prominent during embryonic development, in brain regions with proliferative potential and in haematopoietic tissue (Morland, Rolseth et al. 2002, Kauffmann, Rosselli et al. 2008, Hildrestrand, Neurauter et al. 2009), as well as in multiple cancer forms, in testis and thymus compared with normal tissue. Studies on the mammalian brain have revealed progenitors with the ability to produce new neurons and glial cells (Eriksson, Perfilieva et al. 1998, Taupin and Gage 2002, Kempermann, Gast et al. 2003), and integrate into pre-existing neural circuits (Cameron and Mckay 2001). Neural stem cells, defined with the characteristics of long-term self-renewal and multipotentiality, persist throughout life in various mammalian species, including humans (Temple 2001).

Neural stem cells are mainly located in the sub-ventricular zone (SVZ) of the lateral ventricles and the sub-granular zone (SGZ) of the dentate gyrus of the hippocampus. Neurons differentiated in SVZ migrate through the rostral migratory stream to the olfactory bulb, whereas neurons from the SGZ migrate into the granule cell layer of the dentate gyrus of the hippocampus and become dentate granule cells (Spitzer 2006, Zhao, Deng et al. 2008). The proliferation and differentiation of these cells are required to replace damaged neurons and regain brain function after trauma or hypoxic-ischemic stress. DNA base lesions accumulating during such events are removed by DNA glycosylases in the BER pathway, thus preventing cytotoxicity and mutagenesis. In the brain, the most common oxidative lesions are 8-oxoguanine and FapyG, which arise from oxidation and reduction of 8-hydroxyguanine lesions (Kalam, Haraguchi et al. 2006). Here, one of the DNA glycosylases, Neil3, plays an important role (Sejersted, Hildrestrand et al. 2011).

NEIL3 is also the main DNA glycosylase that removes hydantoins in ssDNA (Liu, Bandaru et al. 2010). The phenotype of mice deficient in *Neil*3 does not appear to be associated with genomic instability, but rather with impaired proliferative capacity of

the neural stem/progenitor cells, in both the SGZ of the hippocampal dentate gyrus and in the sub-ventricular zone (SVZ) of the lateral ventricles (Torisu, Tsuchimoto et al. 2005, Rolseth, Rundén-Pran et al. 2008, Hildrestrand, Neurauter et al. 2009). These mice exhibit learning and memory deficits and show a decreased anxiety-like behaviour (Regnell, Hildrestrand et al. 2012).

Mice lacking Neil2 (*Neil2*$^{-/-}$) appear to be hyper-responsive towards certain inducers of inflammation and display an age-dependent decline in genomic stability (Chakraborty, Wakamiya et al. 2015). A combination of innate inflammation and genomic damage could play a critical role in the development of breast cancer, but also in other diseases as well.

DNA-repair glycosylases overlap in their substrate specificities, especially the mammalian Nei homologs. NEIL1 and NEIL2 have shown repair activity in both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA), and NEIL3 has been shown to exhibit a DNA-binding affinity and AP-lyase activity specific for ssDNA (Takao, Oohata et al. 2009).

The possible roles of BER enzyme activities in protection against prion-induced neurodegeneration had to the best of our knowledge, not previously been explored in mouse models. Therefore, as summarized below, investigating this area was the main objective of our studies.

Aims of the thesis

The main aim of the work presented in this thesis was to explore the role of BER pathway DNA glycosylases in the pathogenesis of prion-induced neurodegeneration. To achieve this, we subjected unique lines of transgenic mice, compromised in their BER enzyme activities, to experimental prion disease. Specifically, we asked whether DNA glycosylases influenced:

- Prion incubation times
- Clinical presentation and duration
- Morphological distribution of pathological lesions, particularly vacuolization
- Brain and spleen distribution of misfolded PrP (PrP^{Sc})
- Accumulation of DNA damage in brain and spleen during the pre-clinical phase and at end-stage prion disease
- Neural progenitor activity at onset and end-stage prion disease
- Brain and spleen mitochondrial respiratory chain activity at onset and end-stage prion disease

We consider investigating these aspects to be important for obtaining a more complete picture of the fundamental processes in neurodegeneration, offering new ways for future prevention and treatment strategies.

Summary of the papers

Paper I

Accelerated clinical course of prion disease in mice compromised in repair of oxidative DNA damage

DNA is constantly under attack by reactive oxygen species, ROS. Oxygen radical-induced damage of proteins, lipids, and nucleic acids is often seen in neurodegenerative disorders. ROS-mediated damage to nucleic acids are mainly repaired by the base excision repair (BER) pathway. This pathway is initiated by DNA glycosylases, which are base lesion-specific enzymes that can scan DNA for chemically altered nucleotide bases. Defects in DNA repair often result in cancer predisposition in rapidly dividing cells or to degeneration and cell death in non-dividing cells, like neurons in the brain. We have studied the development and characteristics of experimental prion disease (RML prions) in double knockout mice, compromised in repair of oxidative DNA damage, *ogg1* $^{-/-}$ and *mutyh* $^{-/-}$, and in isogenic wild-type mice. The double knockout mice are highly cancer prone, with severely shortened life expectancy. Ogg1 initiates removal of the major oxidation product 8-oxoguanine (8-oxoG) in DNA, and Mutyh initiates removal of adenine that has been misincorporated opposite 8-oxoG. The highly reproducible and short incubation period in the mouse-adapted scrapie model (RML prions in C57BL/6Y) allowed the investigation of slow neurodegeneration, before the onset of cancer at around 6-8 months.

The incubation period, pre-clinical phase, was similar in the wild-type and the DNA repair-deficient mice. However, the *ogg1* $^{-/-}$/*muthy* $^{-/-}$ mice displayed a significantly shorter clinical phase. Taking into account the differences in duration of clinical disease, it might be assumed that the regional distribution of prion pathologies could be influenced by reduced BER enzyme activities. As the pathology and lesion profile were similar in the wild-type DNA-compromised mice, it appears that the prion disease affected the same brain regions and progressed similarly in both groups.

The oxidative DNA damage repair does not influence the prion propagation and pathological manifestation, but the accumulation of the lesions may accelerate the final toxic phase of prion disease.

Paper II

Neil3-induced neurogenesis protects against prion disease during the clinical phase

The major pathway for repair of oxidative DNA damage is the base excision repair (BER) pathway, which is initiated by DNA glycosylases. Defects in DNA repair results in DNA damage, and the severity of DNA damage depends on the affected cells. Replicating cells are more likely to acquire mutations, which may lead to malignant disease, whereas terminally differentiated cells accumulate DNA damage, which ultimately leads to cell death and degenerative disease. To elucidate the impact of oxidative DNA damage-induced neurogenesis in prion disease, we have studied the characteristics of experimental prion disease in mice without expression of the DNA glycosylase Neil3 (*Neil3*$^{-/-}$) compared with isogenic controls. In an adult mammalian brain, *NEIL3* is expressed in stem cells of the subventricular zone of the lateral ventricles, subgranular zone of the hippocampal dentate gyrus, and the rostral migratory stream, where neurogenesis occurs. *Neil3* knockout mice display learning and memory deficits and a reduced ability to mount regenerative responses. The prion incubation periods were similar in the wild type and the *Neil3* $^{-/-}$ mice. However, the DNA repair-deficient mice displayed a significantly shorter clinical phase, even though the neuropathology appeared unaffected by Neil3 function. In the brain, the expression of neuronal progenitor markers, nestin, sex determining region box 2, and class III beta-tubulin declined towards end-stage of prion disease. A marker for mature neurons, neuronal nuclei, dropped significantly more in the DNA repair-deficient mice during prion disease, probably due to severe neuronal loss, indicating that neurogenic capacity is impaired in *Neil3* KO mice. Our results suggest that neurogenesis induced by Neil3 repair of oxidative DNA damage protects against prion disease during the clinical phase.

Paper III

Loss of Neil2 promotes the clinical progression of prion disease.

The main pathway for repair of oxidative DNA damage is base excision repair (BER). This pathway is initiated by DNA glycosylases. Neil2 is known to preferentially repair oxidized DNA bases from single-stranded DNA and transcriptionally active genes.

Neil2 knockout mice (*Neil2*$^{-/-}$) display no overt phenotypes, but an age-dependent accumulation of oxidative DNA damage and an increased inflammatory responsiveness. In mice that are intracerebrally inoculated with prions, the prion propagation starts in the germinal follicles of the spleen. We compared experimental prion disease in *Neil2*$^{-/-}$ mice with wild-type mice at disease onset and end-stage with regard to disease progression, accumulation of DNA damage, and mitochondrial respiratory complex activity in brain and spleen. In order to compare the immune responses to prion propagation in the spleen between the two groups of mice, genome-wide RNA sequencing of the spleen, at onset and end-stage prion disease, was performed. The clinical phase of disease progressed more rapidly in the *Neil2*$^{-/-}$ mice compared with the wild-type mice. Levels of relative DNA damage in brain increased in both groups and slightly more in the *Neil2*$^{-/-}$ mice. Transcriptome data from spleen were similar between the groups at onset of disease, with moderate genomic responses. However, at end-stage, a substantial response was evident in the wild-type mice. Our data suggest that Neil2 counteracts toxic signalling in clinical prion disease distinct from gross pathological manifestations and PrP^{Sc} accumulation.

Discussion

Several incurable neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and prion diseases are characterized by accumulation of misfolded protein conformers of self-encoded proteins (Forman, Trojanowski et al. 2004, Jellinger 2009). Certain protein aggregates, possibly consisting of smaller oligomers, severely impact neuronal function and survival, but the mechanisms underlying this are still poorly understood (Aguzzi and Falsig 2012, Alibhai, Blanco et al. 2016). In prion diseases, PrP^{C} is misfolded into pathological conformers, PrP^{Sc}, in a template-assisted manner, which is an assembly of conformers of PrP^{C} (Prusiner 1998). Importantly, by this mechanism, the abnormal protein conformers act as "seeds" for further misfolding and can spread within tissues and between organs, and even, in some instances, between hosts. Interestingly, it has also been observed that some non-prion disease associated proteins can propagate and spread in prion-like style within the CNS, such as alpha-synuclein (Clavaguera, Lavenir et al. 2013), and amyloid-beta (He, Zheng et al. 2017). Currently, there is no evidence that neurodegenerative diseases associated with protein misfolding, except prion diseases, are transmissible diseases under natural conditions. However, in experimental transmissions, seeding and subsequent spread of protein misfolding, much like that observed in prion disease, have been demonstrated (Sørby, Espenes et al. 2008, Aguzzi and Rajendran 2009, Collinge 2016).

Another area of similarity between the chronic neurodegenerative diseases is the impact of reactive oxygen species (ROS)-induced stress in disease development. Normally, ROS are involved in many physiological cellular processes, including intracellular signalling (Ermak and Davies 2002, Ray, Huang et al. 2012), apoptosis, proliferation, and differentiation of embryonic and somatic stem cells (Tsatmali, Walcott et al. 2006, Varum, Momčilović et al. 2009, Nitti, Furfaro et al. 2010, Le Belle, Orozco et al. 2011). However, in some situations, levels are too high for prolonged periods of time, and this may lead to cellular demise. This is particularly well documented in AD, in which extensive oxidative stress and ROS-mediated DNA damage in mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) have been observed early in the disease development, and which can exacerbate disease progression (Lyras, Cairns et al. 1997, Gabbita, Lovell et al. 1998, Wang, Xiong et al. 2005, Wang, Markesbery et al. 2006, Dumont and Beal 2011). Similarly, in the brains

of Creutzfeldt-Jakob disease (CJD) patients, increased levels of oxidative damage to nucleic acids have been observed (Guentchev, Siedlak et al. 2002). Interestingly, stress-induced damage to mtDNA has been shown to shift the differentiation of neural stem cells from neuronal lineages to astrocytes (Wang, Osenbroch et al. 2010, Wang, Esbensen et al. 2011). These studies accentuate the importance of mitochondrial DNA repair and DNA glycosylases to support physiological neurogenesis and differentiation.

Excessive ROS production is counteracted by several antioxidant defence systems, including enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase, and the peroxide scavengers peroxiredoxins (Chae, Kang et al. 1999). It has been reported that patients with AD, probably in response to increased ROS levels, have an increased activity of the antioxidant proteins catalase, SOD, glutathione peroxidase, and glutathione reductase in the hippocampus and amygdala (Pappolla, Omar et al. 1992, Ferrari 2000).

Increased levels of ROS can lead to DNA damage by induction of more than 20 different base adducts. However, guanine is particularly vulnerable and, thus, 8-oxoG is generally considered a sensitive marker of ROS genotoxicity. The base excision repair (BER) pathway enzymes are the main enzymatic machinery for the removal of oxidized bases from mtDNA and nDNA. Therefore, it has been suggested that the increased levels of oxidized base adducts observed in AD could result from compromised BER pathway enzyme activities, particularly of OGG1 that removes 8-oxoG (Mao, Pan et al. 2007). Impairment of BER enzymes could be due to mutations or polymorphisms in BER genes, or could result from disease-associated post-translational modifications of the enzymes. In addition, it might be driven by epigenetic alterations, which could influence transcription of BER genes. Although compromised BER activity in AD and other neurodegenerative diseases have been reported in many studies (Ray, Huang et al. 2012), reviewed in (Hegde, Hegde et al. 2011), the importance of such enzyme deficiencies as main driver in disease development remains to be established.

In the “mitochondrial cascade hypothesis”, it is suggested that relatively high individual and cellular basal levels of mitochondrial ROS production (Swerdlow, Burns et al. 2014) will lead to increased mtDNA damage. Although distinct isoforms

of the same DNA repair proteins that operate within the nucleus are present in the mitochondria (Gredilla, Bohr et al. 2010), this could initiate a vicious cycle, with even higher ROS levels that reinforce further mtDNA damage and mitochondrial dysfunction, ultimately leading to neuronal degeneration (Swerdlow and Khan 2004, Swerdlow, Burns et al. 2014).

The involvement of ROS-mediated DNA damage in prion disease is relatively unexplored. It has been suggested that PrP^C could contribute to protection of human neuronal cells against DNA damage, both under normal conditions and after induced oxidative stress (Senator, Rachidi et al. 2004, Watt, Routledge et al. 2007) or even be directly involved in DNA repair pathways (Bravard, Auvré et al. 2014). Thus, it has been proposed that PrP^C can combat oxidative stress and reduce cell death caused by post-ischaemic reperfusion (Zanetti, Carpi et al. 2014). Given that loss of PrP^C functions occurs as part of prion disease development, this could in itself contribute to increased levels of ROS-mediated DNA damage.

We reasoned that studying prion disease development in mouse models with loss of one or more BER enzymes would be one approach to further explore the impact of ROS-mediated damage to nucleic acids in the disease pathogenesis. To the best of my knowledge, studies involving experimental prion disease in mice with impaired BER-repair capacity with detailed study of disease progression and characteristics including levels of DNA damage, have not previously been reported.

Thus, the primary objective of the studies in this thesis was to characterize prion-disease development in different lines of transgenic mice, all compromised in BER pathway DNA repair enzymes.

In contrast to rodent models of AD (Goedert and Spillantini 2006, Yoshiyama, Higuchi et al. 2007, Selkoe 2011), mouse models of prion diseases have been shown to reproduce all the major features of the disease pathogenesis and neuropathology, as well as strain characteristics of the prion agents (Fraser and Dickinson 1968), reviewed in (Bruce 2003). Transgenic mouse models are also valuable in modelling the so-called species transmission barrier and spectrum for a given prion isolate, by transmission to mice encoding *Prnp* genes from various species, as reviewed in (Béringue, Vilotte et al. 2008).

In our studies, the incubation period, defined as the time from inoculation to the first appearance of clinical signs, as well as all other disease features within the group of wild-type mice, was virtually identical, demonstrating the consistency and reproducibility of this disease model. In contrast to most previous studies, we aimed at separating the pre-clinical incubation period from the phase of clinical disease. Arguably, accurate assessment of disease onset is challenging. Importantly, all inoculation experiments were done in the same mouse facility and animals were observed twice daily by personnel with years of experience in experimental prion disease in mice. Clinical signs were recorded for each animal. Despite this, it is obvious that subtle clinical signs could have been overlooked or misinterpreted. Importantly, this would probably also occur if some groups of animals had displayed atypical signs. As only signs typical for prion disease in mice were observed, we consider our assessment of the disease onset to be adequate approximations for comparisons between groups.

Generally, we observed that the onset of clinical signs, normally first presented as subtle stiffening of the tail, was remarkably similar between all groups of mice. However, the duration of the clinical phase varied significantly, being shorter in all transgenic mice with compromised BER pathway DNA repair enzymes.

Prion infection in mice inoculated with RML, shows that the disease develops in two distinct phenotypic phases. The first phase is a clinically silent phase, dominated by prion propagation until the level of infectivity in the brain reaches a plateau (Sandberg, Al-Doujaily et al. 2011). Astrocytes and microglia initially respond by increasing pro-survival signals (Schultz, Schwarz et al. 2004, Aguzzi, Nuvolone et al. 2013). The activities of phagocytic microglia are, in prion pathogenesis as in other neurodegenerative diseases, considered diverse and not always beneficial. Microglia seem to be able to transport Aβ seeds (Meyer-Luehmann and Prinz 2015) and prions (Baker, Martin et al. 2002, Aguzzi, Barres et al. 2013) throughout the brain, which may lead to worsening of the disease and neurodegeneration. Nevertheless, reduction of microglial phagocytosis is unfavourable; as such activity of microglia is a well-established limiting factor for prion propagation within the brain (Falsig, Julius et al. 2008, Kranich, Krautler et al. 2010). However, further pro-inflammatory activation of microglia can result in augmented inflammatory damage and release of ROS, which will decrease neuronal function and loss of neurons. Although our studies did not

focus primarily on microglia in prion disease, in paper II of the thesis, we compared the levels of mRNA expression of microglia markers *Cd68, Cd86* and inflammation markers *TNFα* and *Il1β* at onset and end-stage of disease. It has previously been shown that the microglial response in Neil3-deficient mice with hypoxic stroke was decreased by 30 % compared with wild-type mice (Sejersted, Hildrestrand et al. 2011). In our study, we found a marked activation of microglia already at onset in both genotypes, which increased even further towards end-stage. Thus, in prion disease, the loss of Neil3 apparently did not compromise microglial proliferation during prion-induced neurodegeneration. Generally, our results fit well with previous observations showing profound astrocytic and microglial activation in prion disease (Aguzzi, Barres et al. 2013, Aguzzi, Nuvolone et al. 2013). However, the astrogliosis in the hippocampus and striatum appeared more prominent in the wild-type compared with that in *Neil3*-deficient mice. This might reflect a reduced capacity to recruit or activate astrocytic proliferation in the *Neil3* KO mice. The following neurotoxic phase is much shorter. The cell-protective signaling probably decreases, whereas the neuronal loss and further mitochondrial and nuclear DNA damage increases.

In our experimental model, we noticed, as expected, that pathological features characteristic for prion disease were present before onset of clinical disease, as has been reported by others (Jeffrey, Goodsir et al. 1997, Jeffrey, Halliday et al. 2000). However, in the neurotoxic phase, neuronal death escalates and is the main driver of clinical symptoms. Bravard et al argue that normal PrP^{C} function is activating DNA repair, and that during prion disease this function is compromised, which might accelerate neuronal cell death (Bravard, Auvré et al. 2014). In our studies, a general observation was that DNA glycosylases appeared to prolong neuronal survival, particularly during the toxic phase of prion disease, and that Neil3 activities provided neuroprotection.

Neurons in different brain areas differ in their vulnerability to ROS-mediated damage. Although most brain neurons can tolerate ROS well, neurons in certain parts of the brain, such as those in the hippocampal CA1 region and cerebellar granule cell layer, are particularly vulnerable (Wang and Michaelis 2010). A central hypothesis in neurodegenerative diseases is that the misfolded protein aggregates spread and distribute to specific neurons in predicted areas of the brain. Different strains of

mouse-adapted scrapie have been reported to target specific brain regions preferentially (Bruce 2003). Therefore, the lesion profile was performed by scoring nine predefined brain areas (Fraser and Dickinson 1968) to investigate whether the regional distribution of prion pathologies could be influenced by reduced BER enzyme activities. As reported in paper I, the *Ogg1*$^{-/-}$*/Mutyh*$^{-/-}$ double knockouts had a very similar lesion profile to that of the wild-type mice. A similar observation was made for the *Neil3*$^{-/-}$ and *Neil2*$^{-/-}$ mice, as reported in papers II and III, although with a tendency of milder vacuolization at disease onset in the hypothalamus of the *Neil2*$^{-/-}$ mice. This data set is, however, limited and it seems reasonable to state that, generally, the lesion profile appeared unaffected by loss of the BER enzymes in our material. Interestingly, we did not observe any compensatory upregulation of mRNA levels of BER enzymes during the course of disease or in response to the knockout in any of our experiments.

Western blot analysis was performed for detection of PrP^{Sc} and characterization of the banding pattern after treatment with proteinase K, which reveals the so-called PrP^{Sc} type, a reproducible characteristic of prion strains. The brain samples were probed with the primary antibody Bar 224 (paper I), which binds to the C-terminal part of PrP or by use of the OIE-approved TeSeE western blot protocol, which also uses a monoclonal antibody (Sha31) that binds to the C-terminal domain of PrP (papers II and III). The banding patterns after proteinase K were similar between the groups, demonstrating that a shift in PrP^{Sc} type had not occurred in any of the knockout lines included in our study. In addition, these data showed semi-quantitatively, that similar levels of PrP^{Sc} accumulated in the brain between the mouse lines.

One question of particular relevance to our studies is whether RML prion disease results in increased levels of oxidative DNA damage, such as 8-oxoG and 5-OHC. In our initial study (paper I), the main focus was on disease development and morphological characterization, and all material was sampled at end-stage. Thus, material for further analyses was unfortunately sparse, but we managed to provide some assessment of nuclear and mitochondrial DNA damage in both brain and spleen, by use of a Real-time PCR-based analysis (Wang, Xiong et al. 2005). The results showed that in brain there was a marked increase in DNA damage in both nuclear and mitochondrial DNA in the *Ogg1*$^{-/-}$*/Mutyh*$^{-/-}$ double knockouts, whereas in the wild-type mice there appeared to be no disease-associated increase in DNA damage. This

limited data set did not reveal any increase in DNA damage levels in the spleen. In paper III, the same method was used (Figure 3A), and here it is evident that there is a strong increase in brain DNA damage levels at both onset and end-stage prion disease in both genotype groups. An increase is also seen in the spleen, particularly prominent in the wild-type mice, which also suffered a much longer clinical phase of disease. The amount of available material of adequate quality for analysis was better in paper III than paper I, and we also had observations at two stages of the disease. Therefore, the data set presented in paper III probably provides a more accurate estimate of accumulation of DNA damage in this disease model.

As reported in papers II and III, we also investigated global levels of DNA damage, by use of mass spectrometry analysis from half brains. This is a well-established method with high accuracy. We do not observe a global increase in levels of 8oxoG nor 5ohdC in brain (paper II and III) or spleen (paper III), neither at onset nor end-stage of RML prion disease. It should, however, be kept in mind that damage to neuronal DNA in those regions most affected by prion toxicity will be diluted, not only by DNA from less affected areas, but also from glia cells that increase in numbers during gliosis. One way of circumventing this challenge would be to analyse material derived from laser-micro dissection from defined areas of the brain. While there are some limitations in global DNA damage analysis, the PCR-based method for DNA damage analysis also has its clear disadvantages. First, it can be challenging to establish and validate and it will provide information of relative DNA damage that affect only the analysed genes. Obviously, extrapolating from this to genomic levels of damage will be risky. Moreover, this method does not provide specific information on the type of DNA lesions, which is relevant. Levels of transcription also influence this method, as it is sensitive to single-stranded DNA. Taken together, our data show that in RML prion disease there is no global increase in oxidative DNA damage, when analysed on whole tissue preparations. However, and despite its limitations, data derived from the PCR-based analysis of DNA damage clearly suggest that significant DNA damage occurs in this disease model, both in brain and in the spleen. Moreover, DNA damage appears to take place both in nuclear and mitochondrial DNA, and increased levels of damage is evident already at the onset of clinical signs, and increases further towards end-stage disease (Lee, Sohn et al. 1999, Guentchev, Voigtländer et al. 2000). Our data correlate with those observed in human cases of

CJD, in which a semi-quantitative assessment revealed very high levels of 8oxoG in brain, particularly in cases with a long disease duration (Guentchev, Siedlak et al. 2002). The levels of 8oxoG seen in some of the CJD cases were similar to or higher than that observed in the few cases of AD included in that study.

In 1928, Ramon Y Cajal stated that no new neurons were generated in the central nervous system after embryonic development (Cajal and May 1928). It was widely accepted that the brain was devoid of any regenerative capacity. However, evidence of new neurons was found in adult rats after brain trauma in the early 1960s (Altman 1962). Several subsequent studies have demonstrated the presence of neuronal progenitors, with the capacity to produce glial and neuronal cells (Taupin and Gage 2002, Kempermann, Gast et al. 2003). In *Neil3* KO mice, however, the proliferation capacity appears compromised (Sejersted, Hildrestrand et al. 2011, Regnell, Hildrestrand et al. 2012). We were therefore interested in analysing RML prion disease development in this KO line, since it could provide new insights into the role of adult neurogenesis in response to prion pathology. In the RML disease model, the hippocampal area is heavily affected. As observed in paper II, whole brain gene expression of neuronal progenitor markers appeared at normal levels at disease onset, but declined towards end-stage. The gene expression level of Doublecortin (Dcx), a neuroblast and immature neuron marker, was increased at end-stage compared with onset of prion disease in wild-type mice. In the *Neil3* KO mice, a similar upregulation was not detected, which supports the role of Neil3 as an activator of neurogenesis during the clinical phase, probably in response to ongoing neuronal loss. In line with this, expression levels of *NeuN*, a marker for mature neurons, dropped significantly, signifying severe neuronal loss. Interestingly, this was somewhat more pronounced in the *Neil3* KO mice than wild-type mice, probably reflecting their compromised neuronal proliferation capacity. In a normal brain, neurons are replaced during normal cell turnover or after brain injury (Kim, Schafer et al. 2006, Ming and Song 2011). Interestingly, it has been observed that in neurodegeneration, compensatory neurogenesis is reduced or even discontinued, and that stem cells of the SGZ of the dentate gyrus can accumulate and replicate prions, which could exacerbate disease progression, as well as to compromise and distort normal neuro-regenerative capacity (Relaño-Ginès, Gabelle et al. 2013).

As mentioned, neurons have extremely high energy demands. The needed energy is generated through mitochondrial oxidation with glucose as the primary energy substrate. It is well-established that gradual dysfunction of neuronal mitochondria during aging or in neurodegeneration is a major pathogenic component (Grimm and Eckert 2017). In order to evaluate mitochondrial function at onset and end-stage prion disease, we measured the enzymatic activity of respiratory complex I and complex V in freshly isolated mitochondria from brain and spleen tissue (paper III). In the brain, the activity of complex I was stable during the progress of disease, whereas the activity of complex V increased somewhat in the wild-type mice at end-stage. Interestingly, in the spleen, the activity of both complexes increased dramatically in the Neil2 KO mice towards end-stage, whereas in the wild-type mice this activation was absent. In search for underlying mechanisms that could explain this observation, and gain further insight of immunological processes, we decided to carry out a genome-wide transcriptome analysis of the spleen at onset and end-stage of prion disease in both groups of mice. At disease onset, there was little difference in differentially expressed genes (DEGs) between the genotypes, with a low number of genes being upregulated and a higher number being downregulated. However, at end-stage, a dramatic increase in DEGs appeared in wild-type animals, but not in the Neil2 KO mice. Further analysis revealed that in the Neil2 KO mice, genes related to proliferation of leukocytes and lymphocytes, and stimulation of mitochondrial function was upregulated, in contrast to the wild-type mice in which these processes were downregulated at end-stage. It is tempting to speculate that the increased mitochondrial activity in the Neil2 KO mice at end-stage could result from cellular proliferation, possibly in combination with mitochondrial fission, but our data do not allow confirmation of this. However, the gross spleen morphology and follicular accumulation of PrP^{Sc} was similar between wild-type and repair-compromised mice. It has earlier been shown that prion propagation (ME7 strain) in spleen (Fraser and Dickinson 1970, Kimberlin and Walker 1988) can lead to changes in splenic follicular architecture (Kim, Han et al. 2016). This was not observed in our study. The proliferative response in spleen and that the $Neil2^{-/-}$ has been shown to be hyper-responsive to certain forms of inflammatory insults (Chakraborty, Wakamiya et al. 2015), indicate that the anti-inflammatory effects of Neil2 contributes protection against prion-induced inflammation. Thus, it seems reasonable that loss of Neil2

results in harmful pro-inflammatory cascades that could at least partly explain the short clinical duration and abrupt clinical end-point in the Neil2 KO mice.

Repair of DNA damage is important during prion diseases. DNA glycosylases prolong neuronal survival during the toxic phase of prion diseases, and probably Neil3-activities provide neuroprotection. Unfortunately, prion diseases are lethal and these enzymes can only prolong the clinical phase. Our observations of similar time of onset of disease suggest that neurogenesis probably has a minor effect in slowing down disease progression in the pre-clinical phase. It is important to expand our knowledge of the mechanisms in neurodegeneration, potentially offering new ways for future prevention and treatment strategies.

Conclusions

Our data show that the general features of prion disease in terms of pre-clinical incubation periods and brain lesion profiles and PrP^{Sc} types are largely unaffected by the loss of BER enzyme activities. These aspects of prion disease appeared very similar in all mouse lines employed in our studies. However, in all BER-compromised mice, we observed a more dramatic and shortened clinical phase of prion disease, clearly suggesting that repair of ROS-induced DNA damage in the cell nucleus and/or in mitochondria contributes, probably in different ways, to neuroprotection during the final toxic phase of prion-induced neurodegeneration. Taking into consideration the relatively short clinical duration of disease in the RML mouse model, the significant differences observed among the mouse lines with regard to clinical duration, suggest quite substantial roles for BER-mediated DNA repair in counteracting toxic signalling in prion disease.

Future perspectives

The studies reported herein represent the first examination of prion disease development in BER pathway-compromised lines of mice. Our studies proved a first glimpse of insight into the roles of BER enzymes in combatting prion-induced neurodegeneration. The results might also be of some relevance for other neurodegenerative disorders associated with protein aggregation. One rather prominent challenge in our studies is that we have analysed a large number of highly relevant parameters; however, at a global scale, since we have studied whole-brain preparations in many of our analyses. The drawback of this is obviously that the prion pathology is not uniformly distributed in the tissue and by analysing whole or half brain preparations, important features linked to the pathogenesis will be diluted in readings from less or even unaffected brain tissue, and thereby go unnoticed. To combat this rather difficult challenge one would need further and expanded studies in which specific areas of brain were analysed after being sampled by laser micro dissection, for instance for gene expression analysis – or by serial sections in combination with *in situ* morphometric techniques that would overcome the dilution effects and allow cell-specific activities to be recorded. On the contrary, when taking into consideration these dilution effects in some of our observations, the differences we do observe, are probably of prominent importance. As such, our studies have opened the field of BER pathway enzyme activities in prion disease and provided the first important clues for further studies in this fascinating field.

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Appendix with papers

Paper I

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Original Contribution

Accelerated clinical course of prion disease in mice compromised in repair of oxidative DNA damage

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ABSTRACT

The detailed mechanisms of prion-induced neurotoxicity are largely unknown. Here, we have studied the role of DNA damage caused by reactive oxygen species in a mouse scrapie model by characterizing prion disease in the *ogg1*$^{-/-}$ *mutyh*$^{-/-}$ double knockout, which is compromised in oxidative DNA base excision repair. Ogg1 initiates removal of the major oxidation product 8-oxoguanine (8-oxoG) in DNA, and Mutyh initiates removal of adenine that has been misincorporated opposite 8-oxoG. Our data show that the onset of clinical signs appeared unaffected by Mutyh and Ogg1 expression. However, the *ogg1*$^{-/-}$ *mutyh*$^{-/-}$ mice displayed a significantly shorter clinical phase of the disease. Thus, accumulation of oxidative DNA damage might be of particular importance in the terminal clinical phase of prion disease. The prion-induced pathology and lesion profile were similar between knockout mice and controls. The fragmentation pattern of protease-resistant PrP as revealed in Western blots was also identical between the groups. Our data show that the fundamentals of prion propagation and pathological manifestation are not influenced by the oxidative DNA damage repair mechanisms studied here, but that progressive accumulation of oxidative lesions may accelerate the final toxic phase of prion disease.

The chemical integrity of DNA, particularly the nucleotide bases, is steadily under attack by reactive oxygen species (ROS) and, despite being shielded by an elaborate system of antioxidants, DNA repair is essential. Oxidative DNA base lesions that cause minor structural changes to DNA are mainly repaired by the base excision repair (BER) pathway [1,2]. Purines undergo oxidation of the ring atoms leading to various chemical modifications. Most notably, the highly mutagenic guanine derivative 8-oxoguanine (8-oxoG) is formed in large quantities. The 8-oxoG lesion has strong miscoding properties and both bacterial and eukaryotic DNA polymerases frequently insert adenine opposite 8-oxoG [3]. BER is initiated by DNA glycosylases, which are base lesion-specific enzymes that scan DNA for chemically altered nucleotide bases among a vast excess of normal bases. Mammalian cells express several DNA glycosylases for removal of oxidized bases, including removal of 8-oxoG and the corresponding ring-fragmented purine formamidopyrimidine derivative faPyG [4–9]. The Mutyh DNA glycosylase is also involved in protection against oxidative damage, although it does not remove oxidatively damaged bases as such [10]. Mutyh removes adenine misincorporated opposite 8-oxoG, which is frequently found as the result of erroneous replication of DNA containing 8-oxoG. Mutyh also removes 2-hydroxy-2-deoxyadenosine, which is a mutagenic lesion occurring by misincorporation of 2-hydroxy-2-deoxyadenosine triphosphate (oxidized dATP) during replication [11]. Inactivation of both Mutyh and Ogg1 in mouse strongly increases the incidence of lung and small intestinal cancer [12,13], supporting a causal role for unrepaired oxidized bases in cancer development. Inherited variants in the human *MUTYH* are associated with somatic mutations in colorectal tumors or adenomas [14,15].

Chronic oxidative stress is thought to be an important element in the pathogenic processes leading to slowly developing diseases such as cardiovascular disease, type 2 diabetes, and several neurodegenerative conditions. Defects in DNA repair often result in cancer predisposition in rapidly dividing cell types, whereas in postmitotic nondividing cells, such as neurons, a gradual decline in function occurs, ultimately leading to degeneration and cell death.

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One commonality for many neurodegenerative diseases is progressive dysfunction and death of neurons in selected areas, associated with aggregates of specific misfolded proteins [16]. These neurodegenerative diseases include Alzheimer disease (AD), Parkinson disease, Huntington disease, and the prion diseases, such as Creutzfeldt–Jakob disease (CJD). In the development of AD, oxidative damage is thought to be particularly important [17]. Increased levels of ROS generate molecular modification of proteins [18], lipids [19], and DNA [20,21], both in the nucleus and in mitochondria. Deposits of amyloid-β (Aβ), which is a pathological by-product of the amyloid precursor protein [22], aggregate and contribute to ROS generation upon binding metals such as Cu^{2+}, Zn^{2+}, or Fe^{2+} [23,24] and, indirectly, by stimulation of mitochondrial ROS production and by initiating an inflammatory response in microglial cells [25]. The involvement of ROS-mediated DNA damage in prion diseases is poorly understood. However, studies of brains of patients with Creutzfeldt–Jakob disease showed increased levels of oxidative DNA damage of nucleic acids [26], in accordance with previous observations of elevated levels of oxidative stress markers in mice experimentally inoculated with prions. In another mouse prion model increased levels of lipid peroxidation were observed at an early stage of the incubation period [27]. Interestingly, an antioxidant function for the prion protein PrP has been proposed, and neuronal cells from mice devoid of PrP have shown increased susceptibility toward oxidative stress [28]. Accordingly, PrP-deficient mice also show dramatically increased levels of oxidative damage upon transient brain ischemia compared with their wild-type counterparts [29–31] Thus, in the development of prion disease, loss of antioxidant scavenging capacity (loss of PrP function) might occur in combination with increased ROS generation. Studies of prion infection in cell cultures have shown that prion infection elicits an acute and transient increase in ROS levels and that in certain subsets of cells with manifest apoptosis enhanced ROS levels were found, correlating with levels of aggregated prion protein (PrP^{Res}) [32].

Studies of Rocky Mountain Laboratories (RML) prion infections in mice have shown that the disease develops in two distinct phases. First there is a clinically silent phase, dominated by prion propagation and buildup of infectivity in the brain that reaches a plateau of variable duration. This is followed by a much shorter neurotoxic phase accompanied by clinical signs [33]. These and several other observations have shown that infectious prions are not instantly toxic to neurons. How the toxic phase of prion disease is initiated is unclear, but the process is dependent upon endogenous cellular PrP (PrP^{C}) expression, suggesting that PrP^{C} might mediate a toxic signaling cascade driven by a late-stage by-product of prion propagation, which, theoretically, can be uncoupled from infectious aggregates.

Here, we report the progression of experimental prion disease in mice with compromised DNA-repair capacity. Our data show that in mice that are deficient in repair of oxidative DNA base lesions experimental prion disease occurs through a much accelerated toxic and clinical phase, while maintaining the overall disease characteristics in terms of pathology and preclinical phase.

Materials and methods

Ethics statement

This study was carried out in accordance with the Norwegian Regulation on Animal Experimentation, which is based upon the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The study was approved by the Committee on the Ethics of Animal Experiments at the National Veterinary Institute and by The Norwegian Animal Research Authority (ID No. 1455).

Mice

For these experiments, 30 mice, 12 C57Bl/6Y (wild-type) and 18 transgenic *ogg1*$^{-/-}$ *mutyh*$^{-/-}$ mice, were inoculated intracerebrally with the RML isolate. The mice were female and weighed around 20 g at inoculation. Two mice in the *ogg1*$^{-/-}$ *mutyh*$^{-/-}$ group died of non-prion-related sickness 1 day and 77 days postinoculation and were removed from further analysis. Furthermore, 6 wild-type mice and 6 *ogg1*$^{-/-}$ *mutyh*$^{-/-}$ mice served as noninoculated age-matched controls for the DNA-damage analysis. Brain and other internal organs from 5 wild-type and 4 *ogg1*$^{-/-}$ *mutyh*$^{-/-}$ mice served as control tissue for histopathological examinations.

The C57Bl/6 *mutyh*$^{-/-}$ and *ogg1*$^{-/-}$ single-knockout mice were generated previously [34–36]. Wild-type C57Bl/6 mice were bred in-house in the same room. Animals were bred in accordance with European regulations FELASA Category C. The genotypes of the mice were routinely tested by a PCR-based protocol upon tissue collection.

Inoculum

The RML isolate used in this experiment was derived from experimental goats previously inoculated with the sheep scrapie brain homogenate SSBP/1. The prion strain has been propagated for many decades in the Rocky Mountain Laboratories in many lines of mice and adapted into C57Bl/6Y.

Experimental design

All mice, except controls, were anesthetized and inoculated with 20 μl of a 10% inoculum into the right midhemisphere. After the inoculation, the mice were monitored daily and recorded for signs of clinical disease such as ataxia, ruffled coat, hunched back, pelvic limb weakness or paresis, trembling, and lethargy.

Mice were sacrificed at a predefined clinical endpoint and the brain and other internal organs (gastrointestinal tract, liver, spleen, kidneys, heart, and lungs) were collected. The brain was cut longitudinally and one half was fixed in 10% neutral-buffered formalin. Upon fixation, the brain tissue was trimmed into five transverse sections as described by Bruce and co-workers [37] and processed for histopathological analysis and immunohistochemical detection of prion protein (PrP^{Sc}) and the astrocyte marker glial fibrillary acidic protein (GFAP). The other half of the brain was frozen for biochemical analysis and detection of PrP^{Sc} by Western blot.

Immunohistochemistry

After fixation, tissue sections were dehydrated and embedded in paraffin. For detection of PrP^{Sc}, the sections were immersed for 30 min in 98% formic acid, washed, and autoclaved while immersed in 0.01 M citric acid, pH 6.1, for another 30 min at 121 °C. Sections were exposed to a mild proteinase K digestion (4 μg/ml) at 37 °C for 5 min. A cocktail of primary antibodies (F89/160.1.5 1/2000 and 2G11 1:400) was applied for 1 h at room temperature and a commercially available kit (EnVision+ System–HRP (AEC) Mouse; DAKO) was applied for visualization of PrP^{Sc}. Staining for GFAP was performed using a polyclonal rabbit anti-bovine GFAP antibody (Dakopatts) at 1:2000 dilution for 1 h at room temperature and detection by use of the EnVision+ System–HRP (AEC) Rabbit kit (DAKO). The following brain areas were analyzed to generate the lesion profile: dorsal half of the

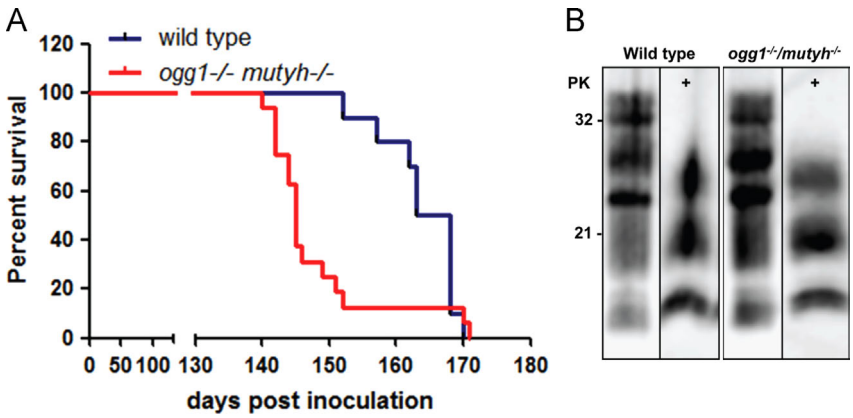

Fig. 1. (A) Survival plot of wild-type and DNA repair-compromised mice ($ogg1^{-/-}$ $mutyh^{-/-}$) after inoculation with RML prions. Clinical signs appeared simultaneously around 138 dpi, but the BER-pathway-deficient mice reached clinical end-stage much faster ($p < 0.05$, log rank Mantel Cox test) than wild-type mice. (B) Western blot of brain PrP, probed with the mAb Bar224, which binds to the C-terminal domain of PrP. The banding pattern after treatment with proteinase K is similar between wild-type and DNA-repair-deficient mice.

medulla oblongata, cerebellar cortex of the folia, cortex of the superior colliculus, hypothalamus, thalamus, hippocampus, septal nuclei, cerebral cortex dorsal to the corpus callosum, and cerebral cortex dorsal to the septal nuclei. The degree of vacuolization was scored from 0 (no vacuoles) to 5 (dense vacuolization).

Western blot analysis for detection of PrP^{Sc}

Brain samples were homogenized at room temperature in a buffer containing Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% (w/v) Triton X-100, 0.5% sodium deoxycholate and treated with proteinase K (PK; 4 μg/ml) for 1 h at 37 °C or with peptide *N*-glycosidase F (PNGase F; New England Biolabs) for 2 h at 37 °C for deglycosylation. Samples were boiled in SDS sample buffer and run on 12% precast polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes by a semidry blotter. Membranes were blocked for 1 h in 5% fat-free dry milk, before incubation with primary antibody Bar224 (Spi Bio, France) overnight at 4 °C. Secondary antibody (goat anti-mouse) labeled with alkaline phosphatase (GE Healthcare, UK) was used to visualize bands with a fluorescence imager (Typhoon 9200; GE Healthcare) after incubation with the ALP substrate (ECFTM, Western blotting reagent pack; GE Healthcare). In addition, an EU- and OIE-approved test (TeSeE Western Blot; Bio-Rad) was used according to the recommendations of the manufacturer. The immunodetection was performed using the monoclonal antibody SHa31, which recognizes the amino acids YEDRYYRE, corresponding to codons 145–152 in human PrP. Peroxidase activity was revealed using an enhanced chemiluminescence (ECL; GE Healthcare) substrate.

DNA damage detection

Real-time PCR-based analysis of DNA damage inhibition of restriction enzyme cleavage was performed as described previously [21]. Briefly, tissues were homogenized with FastPrep-24 (MP Biomedical) and genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen). *Taq*$^{\alpha}$I restriction enzyme digestion and subsequent real-time PCR were carried out with 30 and 6 ng total DNA for nuclear and mitochondrial DNA damage detection, respectively. Relative amounts of PCR products were calculated by the comparative ΔC_T method. The following primers were used: *Gapdh* forward, 5′-CTTCAACAGCAACTCCCACT and reverse, 5′-AAAAGTCAGGTTTCCCATCC; *Tbp* forward, 5′-GGCATCAGATGTGCGTCA and reverse, 5′-CGCAGAAACCTAGCCAAACC; and *mt-Rnr1* forward, 5′-ACTCAAAGGACTTGGCGGTA and reverse, 5′-AGCCCATTTCTTCCCATTTC.

Results

Before inoculation, brain PrP^{C} levels, glycosylation, and proteolytic processing were analyzed by Western blots with two monoclonal antibodies, in wild-type and $ogg1^{-/-}$ $mutyh^{-/-}$ mice, to ensure similar steady-state levels of PrP (Supplementary Fig. 1).

Disease progression

Mice inoculated with RML prions developed neurological signs associated with transmissible spongiform encephalopathy disease and accumulated proteinase-resistant PrP in the brain. Two mice were removed from the experiment; one died 1 day after inoculation owing to complications and one died 77 days postinoculation of unrelated disease. The first signs of clinical disease appeared around 138 days postinoculation (dpi) in both groups of animals, manifest as hunched back, pelvic limb weakness, and lethargy. However, in the DNA repair-compromised group, $ogg1^{-/-}$ $mutyh^{-/-}$, more than 50% of the animals died within a week (145 dpi) after initial symptoms, whereas this point was not reached until day 165 dpi for the wild-type mice (Fig. 1A). Western blots for PrP and PK-resistant PrP fragments revealed no differences between wild-type mice and the $ogg1^{-/-}$ $mutyh^{-/-}$ group (Fig. 1B). Two mice in the $ogg1^{-/-}$ $mutyh^{-/-}$ group, however, experienced a clinical course more similar to that of the wild-type mice, among which 50% of the animals were still alive 2.5 weeks after the first clinical signs.

Histopathology

The severity and distribution of vacuolation were scored in nine different brain areas using blinded samples. These data were used to design lesion profiles (Fig. 2) for both the DNA-repair-compromised animals and the wild-type mice according to [38]. The lesion profiles for wild-type and $ogg1^{-/-}$ $mutyh^{-/-}$ mice were similar, with overlapping confidence intervals.

Vacuolar changes were most prominent in the thalamus, hippocampus, and cerebral cortex and were accompanied by intense PrP^{Sc} deposits, mainly in a stellate pattern [39], and less labeling in the neuropil (Fig. 3, first column). Serial GFAP-labeled sections mirrored the stellate PrP^{Sc} labeling, being marked in some areas, such as the hippocampus, with moderate labeling in other areas such as the cerebellum (Fig. 3, second column). In the cerebellum there was some neuropil-associated PrP^{Sc} labeling. A range of other internal organs was also examined and revealed that two mice in the DNA-repair-compromised group that had been inoculated with RML prions had early lymphosarcoma, and

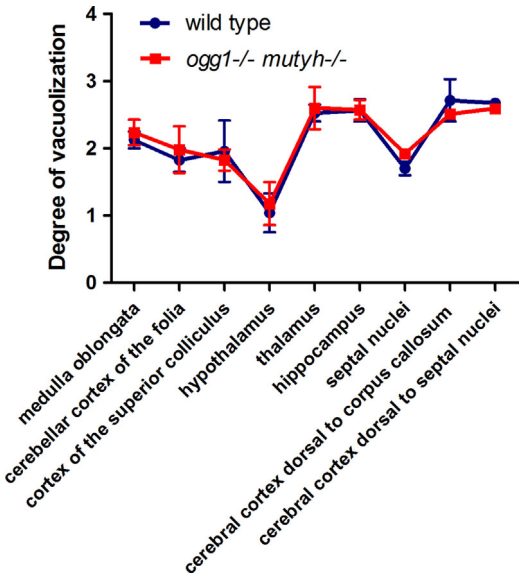

Fig. 2. Distribution of brain lesions in DNA-repair-compromised mice ($ogg1^{-/-}$ $mutyh^{-/-}$) and wild-type at end-stage RML prion disease. Vacuolization scores: 0, no vacuoles; 1, few vacuoles unevenly distributed; 2, few vacuoles evenly distributed; 3, moderate numbers of vacuoles evenly distributed; 4, many vacuoles with some confluence; 5, dense vacuolization.

one DNA-repair-compromised control mouse (not inoculated) had a hemangioma.

DNA damage analysis

Nuclear and mitochondrial DNA from brain of $ogg1^{-/-}$ $mutyh^{-/-}$ mice contained more damage at the endpoint of the clinical phase than wild-type mice. Samples of brain genomic DNA from uninfected mutant and wild-type mice at the same age showed no difference in accumulation of DNA damage (Fig. 4A). It thus seems that accumulation of oxidative DNA damage accelerates the toxic phase of prion disease. Despite prion replication at end-stage in spleen, as judged by accumulation of proteinase-resistant PrP (Supplementary Fig. 2), in this tissue we did not detect any difference in DNA damage between wild-type and $ogg1^{-/-}$ $mutyh^{-/-}$ animals (Fig. 4B). This observation indicates that the genotoxic effect of prion replication is less prominent in the spleen as opposed to brain.

Discussion

BER [1,2] is the major pathway for removal of DNA bases damaged by reactive oxygen species, in both the nucleus and the mitochondria. The ring structure of purines, such as guanine,

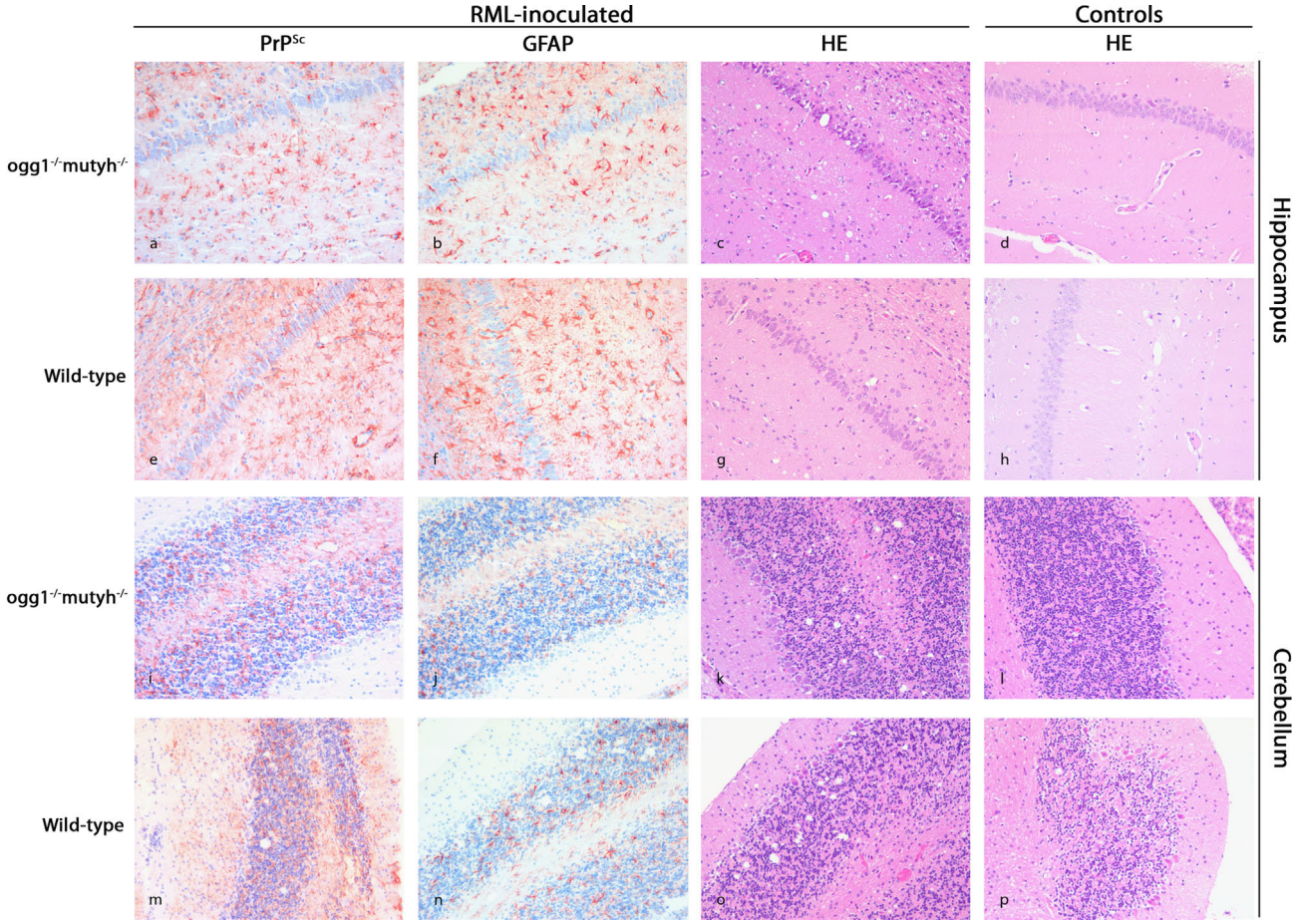

Fig. 3. (a, e, i, m) PrP^{Sc} and (b, f, j, n) glial fibrillary acidic protein (GFAP) labeling and histopathology in (c, g) hippocampus and (k, o) the cerebellar cortex of the folia in $ogg1^{-/-}$ $mutyh^{-/-}$ mice and wild-type mice at clinical end-stage of RML prion disease. Hematoxylin and eosin (HE) sections of wild-type mice are given as controls (d, h, l, p). There is diffuse and marked stellate PrP^{Sc} labeling (a) and stellate GFAP labeling of astrocytes in serial section (b) and moderate vacuoles evenly spread and marked astrocytosis (c) in the hippocampus. Wild-type mice (e–g) show the same changes as $ogg1^{-/-}$ $mutyh^{-/-}$ mice (a–c). Stellate PrP^{Sc} labeling is evident in the granule cell layer, less prominent in the Purkinje cell layer and white matter of the cerebellar folia (i). There is also some punctate neuropil labeling in the same areas. The molecular layer is spared. Image (j) is a serial section showing moderately stellate GFAP labeling of astrocytes in the granule cell layer, and white matter, and sparse stellate labeling in the Purkinje cell layer. The molecular layer is negative. There are some vacuoles in the granule cell layer (k). In the cerebellar folia of wild-type mice a punctate and stellate PrP^{Sc} labeling of all cell layers is observed (m), whereas a stellate pattern of GFAP labeling is mainly found in the granule cell layer and less in white matter (n). In the granule cell layer a moderate number of vacuoles were present, whereas few vacuoles were observed in the molecular layer (o). Original magnification: 200 ×.

is frequently oxidized, resulting in the mutagenic derivative 8-oxoG. This derivative has miscoding properties and DNA polymerases incorporate adenine opposite 8-oxoG. The BER pathway is initiated by DNA glycosylases that scan DNA for altered bases and initiate their removal. Notably, oxygen-derived DNA damage has been shown to be of pathological importance in many other conditions as well, such as type 2 diabetes and cardiovascular diseases (for review see [40]).

Oxidative damage of proteins, lipids, and nucleic acids is often evident in neurodegenerative disorders. Such damage is particularly prominent in conditions associated with mitochondrial dysfunction leading to increased generation of ROS and in maladies in which metal-binding protein fragments accumulate, as these also increase the level of ROS, such as Aβ-containing deposits in AD. Interestingly, the prion protein, which accumulates in a misfolded conformation (PrP^{Sc}) in brain areas affected by prion disease, also binds divalent metal ions with high affinity, suggesting that such protein accumulations could increase the oxidative stress. The direct contribution of ROS to the pathogenesis of prion diseases is somewhat less explored; however, analysis of brain areas in humans affected by CJD showed increased levels of oxidative DNA damage [26]. Interestingly, an antioxidant function has been ascribed to the cellular prion protein PrP^{C} [29–31]; thus in prion disease it is possible that there is a combined effect due to both increased ROS generation and reduced scavenging capacity due to local loss of PrP^{C} function.

Animal models that are suitable for experimental analysis of ROS-mediated DNA damage in slowly developing neurodegenerative diseases are not easily developed, partly because animals with compromised DNA repair are prone to cancer. Therefore, experimental prion diseases with mouse-adapted prion strains might prove valuable because the experimental challenge can be performed early in life and, with incubation periods of 3–5 months, a readout can be achieved before the onset of cancer. The mouse model employed in this study, which carries a combined knockout of *ogg1* and *mutyh,* illustrates this point, as cancer normally develops in these animals after 6–8 months of age, whereas the end-point after prion challenge is reached at about 6.5 months. Accordingly, in our material, only three mice with compromised DNA repair showed early signs of cancer.

As the first signs of clinical prion disease developed simultaneously in wild-type and $ogg1^{-/-}$ $mutyh^{-/-}$ mice, the incubation period for RML prion disease appeared to have been unaffected by the loss of the BER enzymes Ogg1 and Mutyh. However, shortly after the first signs of illness, the DNA-repair-compromised mice suffered a dramatic worsening of their clinical condition and within 1 week more than 50% of the animals were dead. Thus, on average, the clinical time course of prion disease was more than halved in mice with compromised DNA repair, compared with isogenic wild-type mice. Western blot analysis of partially proteinase-resistant PrP revealed (Fig. 1B) that the banding pattern (PrP^{Sc} type) was identical between the two groups of mice, showing no signs of PrP^{Sc} type differences or “strain” switch between the groups.

Taking into account the marked difference in duration of clinical disease, we anticipated that the degree of histopathological changes and gliosis would differ between the groups. However, this seemed not to be the case. The amount and distribution of PrP^{Sc} also appeared very similar in the two groups, corroborating the Western blot data, suggesting that the fundamentals of prion replication and associated pathology were virtually unaffected by the loss of the BER pathway enzymes Ogg1 and Mutyh.

Because neurons in different brain areas differ in their vulnerability to ROS-mediated damage [41], it might be assumed that the regional distribution of prion pathologies could be influenced by reduced BER enzyme activities. To investigate this, standardized lesion profiles were generated by scoring the histopathological hallmarks of prion disease in nine predefined brain areas [38].

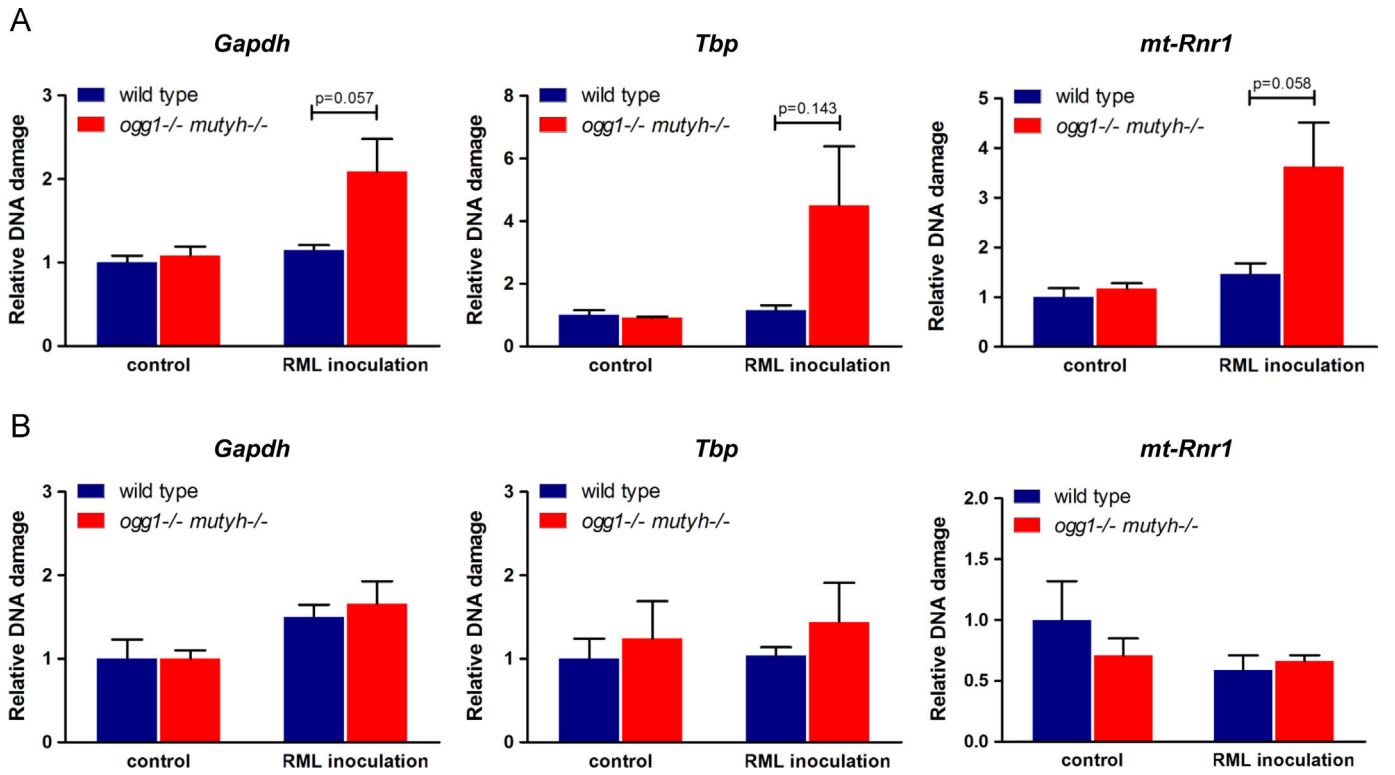

Fig. 4. Level of DNA damage in (A) brain and (B) spleen at end-stage RML prion disease, mice about 200 days of age. Nuclear DNA damage was assessed by analysis of the coding region of the housekeeping genes *Gapdh* and *Tbp* ($n=3$–6), whereas mitochondrial DNA damage was analyzed using the *mt-Rnr1* gene ($n=3$–6). Columns show mean values with SEM.

As the lesion profile was identical between the groups, it appears that the prion disease affected the same brain regions and progressed similarly in both groups.

It is interesting to note that, despite a dramatically different clinical phase of the disease, the histopathological and molecular characteristics, at end-point, are almost identical, at least at light-microscopy levels. Thus, according to our data, the morphological end-point of prion disease provides little information about the rapidity of clinical deterioration in the toxic phase of prion disease [33]. Because of the very short clinical course observed in the DNA-repair-compromised animals, it is tempting to speculate that many of the pathological changes would have been already present at the onset of clinical signs. Further experiments are needed to clarify this and the precise mechanisms causing the clinical signs. There are striking parallels between our observations and a previous report of RML prion disease in mice devoid of heat shock factor 1 (HSF1 KO) [42], in which onset of clinical signs was similar in HSF1 KO mice and controls, but with a much shorter clinical duration in the HFS1 KO group, suggesting that HSF1 provided protection during the clinical phase of the disease, without influencing the preclinical disease progression. The neuropathological changes and proteinase-resistant PrP profiles were also similar between the groups, similar to our observations. It has previously been documented in cell culture experiments that prion replication selectively modifies cellular stress responses [43]. Whether the BER enzymes studied here and HSF1 are components of the same line of defense against prion toxicity remains an intriguing possibility that requires further study.

Our data suggest that during the clinical phase of prion disease, BER of oxidative DNA damage in neuronal cells counteracts ROS-mediated stress and that loss of these enzyme activities greatly accelerates the final phase of prion disease. Many previous studies have identified genetic elements that influence the incubation period of experimental prion disease [44,45], but to our knowledge this report is the first demonstration that DNA repair enzymes specifically influence the rapidity of progression of clinical prion disease, but without altering the incubation period and other disease characteristics. Our data provide support for the theory that prion disease occurs in two distinct phases and that BER of oxidative DNA base lesions is of importance in the clinical, toxic phase of prion disease.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2013.11.013.

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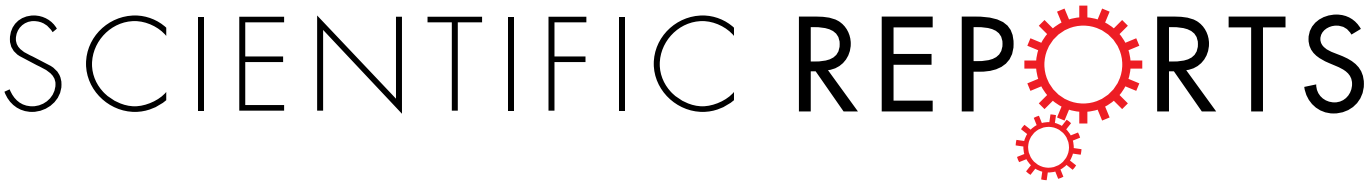
Paper II



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Neil3 induced neurogenesis protects against prion disease during the clinical phase

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Base excision repair (BER) is the major pathway for repair of oxidative DNA damage. Mice with genetic knockout of the BER enzyme *Neil3* display compromised neurogenesis in the sub-ventricular zone of the lateral ventricle and sub-granular layer of the dentate gyrus of the hippocampus. To elucidate the impact of oxidative DNA damage-induced neurogenesis on prion disease we applied the experimental prion disease model on Neil3-deficient mice. The incubation period for the disease was similar in both wild type and *Neil3*$^{-/-}$ mice and the overall neuropathology appeared unaffected by Neil3 function. However, disease in the *Neil3*$^{-/-}$ mice was of shorter clinical duration. We observed a mildly reduced astrogliosis in the hippocampus and striatum in the Neil3-deficient mice. Brain expression levels of neuronal progenitor markers, nestin (*Nestin*), sex determining region Box 2 (*Sox2*), Class III beta-tubulin (*Tuj1*) decreased towards end-stage prion disease whereas doublecortin (*Dcx*) levels were less affected. Neuronal nuclei (*NeuN*), a marker for mature neurons declined during prion disease and more pronounced in the *Neil3*$^{-/-}$ group. Microglial activation was prominent and appeared unaffected by loss of Neil3. Our data suggest that neurogenesis induced by Neil3 repair of oxidative DNA damage protects against prion disease during the clinical phase.

Prion diseases such as Creutzfeldt-Jakob disease in humans and scrapie in sheep and goats are invariably fatal, neurodegenerative diseases characterized by protein misfolding and aggregation. The host-encoded cellular prion protein (PrP^{C}) misfolds into disease-provoking multimeric aggregates, some of which constitute infectious prions[1]. Proteinase resistant remnants of misfolded prion protein are known as PrP^{Sc} (scrapie). Neuropathological hallmarks of prion disease include tissue vacuolization, deposits of misfolded conformers of PrP, reactive gliosis and neuronal loss[2–4]. Experimental prion diseases in rodents replicate all aspects of naturally occurring prion disease, and are therefore valuable models of protein misfolding associated neurodegeneration. Intracerebral inoculation with the mouse-adapted Rocky Mountain Laboratories (RML) scrapie strain have shown that peak infectivity is reached midway into the asymptomatic incubation period, while glial activation and vacuolization gradually develop, particularly in the thalamus and hippocampus, during the second half of the incubation period[5]. Significant involvement of the hippocampus is a well-known feature of other murine prion models as well[6] and prion replication has been shown to occur in neural stem cells (NSC) of the sub-granular layer (SGL) of the dentate gyrus of the hippocampus[7]. The SGL is a major site for adult neurogenesis, responding to various insults like ischemia. The role of neurogenesis in protein misfolding associated neurodegeneration, such as Alzheimer's disease (AD), Parkinson's disease, Huntington's disease and prion diseases is not clarified, and data from different model systems sometimes conflict. For instance, both increased[8] and decreased[9] hippocampal neurogenesis have been reported in AD. Furthermore, a stage-dependent profile has also been observed, with increased neurogenesis at early stages of neurodegeneration[10], followed by a decline in stem cell activity at later stages of severe neurodegeneration[11]. The profound neurodegeneration that occurs in the hippocampus of RML-induced prion disease makes this a useful model for studying SGL-driven neurogenesis in a protein misfolding neurodegenerative disease. In a previous study[12], we examined the development of RML prion disease in mice with combined knockout of DNA repair enzymes, Mutyh and Ogg1, which initiate base excision repair

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(BER) of reactive oxygen species (ROS)-mediated DNA damage[13]. The BER pathway is initiated by DNA glycosylases recognizing modified bases[14], including the mammalian NEIL family, where the three members (Neil 1, 2, 3)[13,15,16] are homologous to the *E. coli* formamidopyrimidine DNA glycosylase and endonuclease VIII (Nei) enzymes[17,18]. The full spectrum of physiological activities of the Neil enzymes has not been clarified and recent data suggest roles beyond DNA repair and genomic stability, possibly related to gene regulatory activities[19,20]. *In vitro* Neil1 and Neil2 display repair activity on both double-stranded DNA and single-stranded DNA (ssDNA) substrates. Neil3 exhibits DNA glycosylase activity and AP-lyase activity specific for ssDNA[21] and is considered the main DNA glycosylase for removal of hydantoins in ssDNA[22]. The phenotype of mice that are deficient in Neil3 is associated with impaired proliferative capacity of the neural progenitor cells[19,20]. In mouse, *Neil3* is highly expressed in neural stem cells, such as SGL and the sub-ventricular zone[23–25]. Interestingly, *Neil3*$^{-/-}$ mice have a reduced capacity for hippocampal neurogenesis after hypoxia-induced neuropathology[19]. Indeed, Neil3-dependent DNA repair appears essential for maintenance of neural stem cell proliferative capacity, which indicates that repair of oxidative DNA damage in NSCs is required for adult neurogenesis[20]. Surprisingly, Neil3 deficient mice showed no change in steady state levels of oxidative DNA damage and genome integrity, indicating a role beyond canonical BER. It thus, appears that Neil3 deficient mice are an interesting model for studying the impact of impaired neurogenesis in neurodegenerative disease. Therefore, in order to broaden our understanding of hippocampal neurogenesis during neurodegeneration, we report a study of prion disease in mice with genetic knockout (KO) of *Neil3*.

Results

All RML-inoculated mice developed neurological signs, and prion disease was confirmed by histopathological analysis. The first signs of clinical disease, kyphosis, pelvic limb weakness and lethargy, appeared around 138 days post inoculation (dpi) in both groups. We observed that *Neil3*$^{-/-}$ mice had a shorter clinical phase than the wild-type mice, with mean survival times from onset to end-stage being 18 and 22 days for *Neil3*$^{-/-}$ and wild-type mice respectively ($p = 0.04$). Kaplan Meier survival plots also revealed statistically different survival curves (Fig. 1A). One of the *Neil3*$^{-/-}$ mice, however, had a more prolonged clinical course, similar to that of the wild-type mice, in which more than 50% of the animals were still alive 18 days after onset of disease.

Analysis of steady state levels of PrP^{C} in brain by western immune blots revealed similar levels of predominantly di-glycosylated PrP^{C} and a similar level of proteolytic processing, as judged by full length (FL) and C-terminal fragment 1 (C1) between the groups (Fig. 1B, left panel). Proteinase K resistant PrP banding patterns (PrP^{Sc} type) were similar at onset and end-stage in both groups (Fig. 1B, right panel).

Distribution of vacuolization in 9 brain areas at onset (Fig. 1C) and end-stage (Fig. 1D) were most prominent in the cerebral cortex, the thalamus and hippocampus as expected for the RML prion strain and in agreement with[26,12].

Expression of DNA glycosylases, *Neil1*, *Neil2*, *Neil3*, *Nth1* and *Ogg1,* in brain at onset and at end-stage prion disease, relative to expression in un-inoculated age-matched controls of wild-type mice, is given in Fig. 2. There was a significant decrease in expression at either onset or end-stage or both for some of the DNA glycosylases (Neil1, Neil2 and Ogg1) in wild-type mice, demonstrating a lack of compensatory up-regulation of brain DNA glycosylases in prion disease.

Accumulation of the oxidative DNA base lesions 8-oxoG and 5-OHC at onset and end-stage prion disease was measured by mass spectrometry. The results, presented in Fig. 3, show no global increase of these lesions during prion disease or differences between the genotypes, indicating that Neil3 is not affecting the steady state level of 8-oxoG and 5-OHC, in which 5-OHC is substrate for Neil3. It thus appears that the Neil3 DNA glycosylase is not important for removing oxidative DNA base lesions genome-wide during prion disease.

Vacuolization was present in both groups and progressed similarly through the clinical phases. There was loss of pyramidal neurons already at onset of disease in both wild-type and *Neil3*$^{-/-}$ mice (Figs 1C and 4B) leading to a reduced thickness of the layer compared with controls. This neuronal loss was accompanied by vacuolization and astrogliosis (Fig. 4A, right panels), covering all layers of the hippocampus. The astrogliosis appeared more prominent in the wild-type mice than the *Neil3*$^{-/-}$ mice in both hippocampus and striatum (Fig. 4C).

The expression of neuronal progenitor markers Nestin (*Nestin*), Sex determining region Y box (*Sox2*) and Class III beta-tubulin (*Tuj1*), were all maintained at normal levels at onset of disease, however, declining towards end-stage (Fig. 5A,B,D). Doublecortin (*Dcx*), a neuroblast and immature neuron marker, was significantly up-regulated at end-stage compared with onset in wild-type mice (Fig. 5C), while the mature neuron marker, Neuronal Nuclei (*NeuN*), was decreased in wild-type and Neil3 KO mice at onset and end-stage of prion disease, due to severe neuronal loss (Fig. 5D). At onset, expression of *NeuN* was significantly lower in *Neil3* KO mice compared with wild-type. Microglial activation was assessed by expression levels of Clusters of differentiation 68 and 86 (*Cd68* and *Cd86*), tumor necrosis-factor alpha (*TNFα*) and interleukin 1-beta (*Il1ß*) (Fig. 6A–D). All genes were markedly upregulated at onset of disease and continued to rise towards end-stage prion disease, except *Il1ß* that declined slightly towards end-stage.

Discussion

The mouse Neil3 DNA glycosylase is required for maintenance and differentiation of neuronal stem progenitor cells during ageing and in response to acute hypoxia/ROS-mediated stress[19,20]. Studies in rat demonstrated that genetic knockdown of the Ogg1 and Neil3 DNA glycosylases mitigates pluripotency and initiates premature senescence of multipotent neural progenitor cells[27]. In this study, we demonstrate that Neil3 protects the brain during the clinical phase of prion disease. The full impact of neurogenesis as a counteracting homeostatic mechanism in slowly developing neurodegenerative conditions, such as AD and prion diseases, has not been clarified. Since experimental prion diseases in rodents are considered *bone fide* prion diseases, reproducibly recapitulating all major aspects of naturally occurring prion disease, we wanted to compare overall disease development and

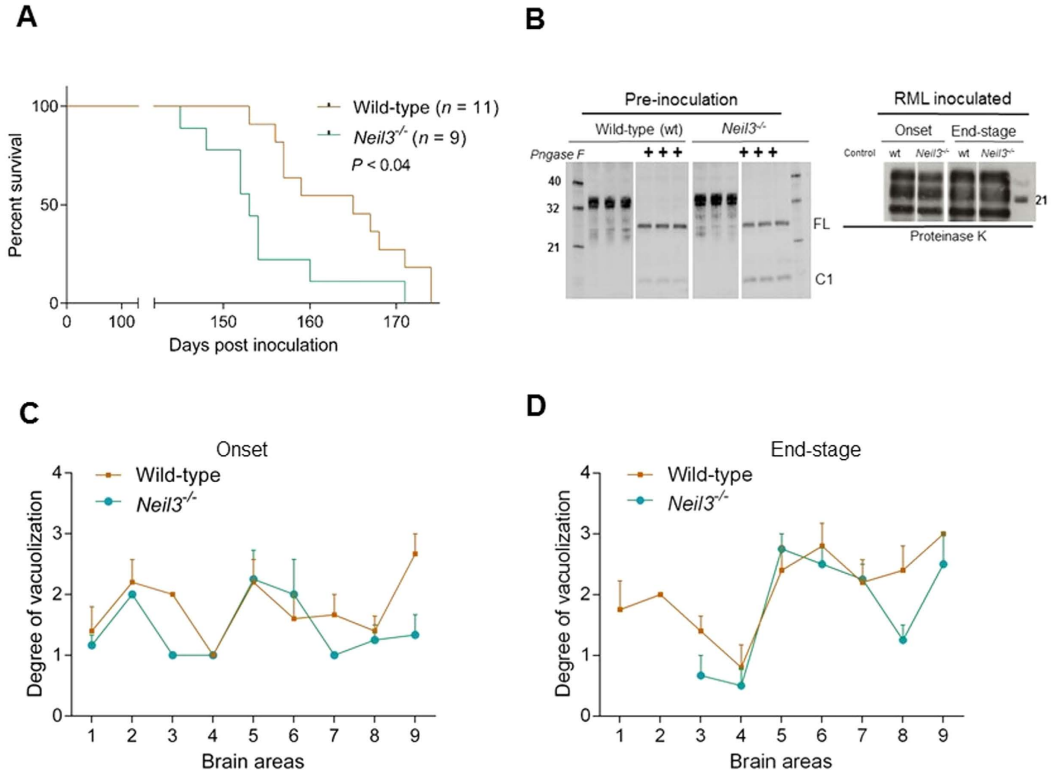

Figure 1. (**A**) Survival plot of wild-type ($n = 11$) and *Neil3*$^{-/-}$ ($n = 9$) mice after intracerebral inoculation with the Rocky Mountain Laboratories (RML) strain of prions. The preclinical phase lasted for around 138 days in both groups of animals, but the *Neil3*$^{-/-}$ mice suffered a shorter clinical phase of disease ($p < 0.05$, Log rank Mantel Cox test). (**B**) Western immune blots of whole brain PrP pre inoculation (left panel, mAb Bar224) and proteinase resistant PrP^{Sc} at onset and end-stage (right panel, mAb SHa31). The steady-state levels of PrP at pre-inoculation was similar between the groups and dominated by di-glycosylated PrP. Levels of full length (FL) the C-terminal truncation product (C1) was also similar between the groups. The banding pattern of PrP^{Sc} as seen after PK treatment was also similar between the groups at both onset and end-stage of disease. (**C**,**D**) Lesion profile. Distribution of brain vacuolization in nine defined areas (1 medulla oblongata, 2 cerebellar cortex, 3 cortex of the superior colliculus, 4 hypothalamus, 5 thalamus, 6 hippocampus, 7 septal nuclei, 8 cerebral cortex dorsal to corpus callosum, 9 cerebral cortex dorsal to septal nuclei) of the brain as observed at onset (**C**) and at end-stage (**E**) in wild-type ($n = 5$) and Neil3 ($n = 4$–6) KO mice. Vacuolization scores: 0 no vacuoles, 1 few vacuoles unevenly distributed, 2 few to moderate number of vacuoles evenly distributed, 4 many vacuoles with some confluence, 5 dense and confluent vacuolization.

characteristics in normal and neurogenesis-compromised mice, like the Neil3 KO line. The involvement of hippocampal SGL early in the pre-clinical disease development and the subsequent severity of neuronal death in this brain area in RML prion disease renders this a particularly well-suited disease model for studying SGL neurogenesis in the face of prion-driven neuronal death.

We have previously studied RML prion disease in mice severely deficient in DNA repair, by combined knockout of Ogg1 and Mutyh and observed that several disease features appeared unaffected by the DNA repair deficiency[12]. However, the clinical phase of the disease was more severe and shorter in the repair-compromised mice, suggesting that DNA integrity is challenged in the clinical, toxic phase of prion disease. Interestingly, data presented here show that the Neil3 deficient mice share several similarities with the Ogg1 and Mutyh double knockouts, with a shorter and more dramatic clinical disease, but without other major alterations in disease characteristics, such as distribution of brain lesions. Despite the fact that DNA repair capacities appears to be gradually overwhelmed during prion disease, neither this nor the lack of Neil3 elicit any compensatory increase in expression levels of other DNA glycosylases with overlapping substrate specificities (i.e. Neil1 and Neil2).

It has been shown that during prion disease, neuronal stem cells are heavily affected and accumulate PrP^{Sc} and could thus potentially contribute to disease progression[7]. Importantly, it was also shown that prion disease skewed the neuronal fate of neuronal progenitors, with loss of neuroblasts and immature neurons and an increase in astrocytes. Thus, in prion disease, adult capacities for neurogenesis are distorted and overrun by neuronal loss. This effect could even out differences in neurogenesis between wild-type and Neil3-deficient mice, at least during the toxic/clinical phase of prion disease. However, our observations of similar time of onset of disease suggest that neurogenesis probably has a minor effect in halting disease progression also in the pre-clinical phase. Gomez-Nicola *et al.* suggest a biphasic model of neurogenesis, with a significant increase in the number of

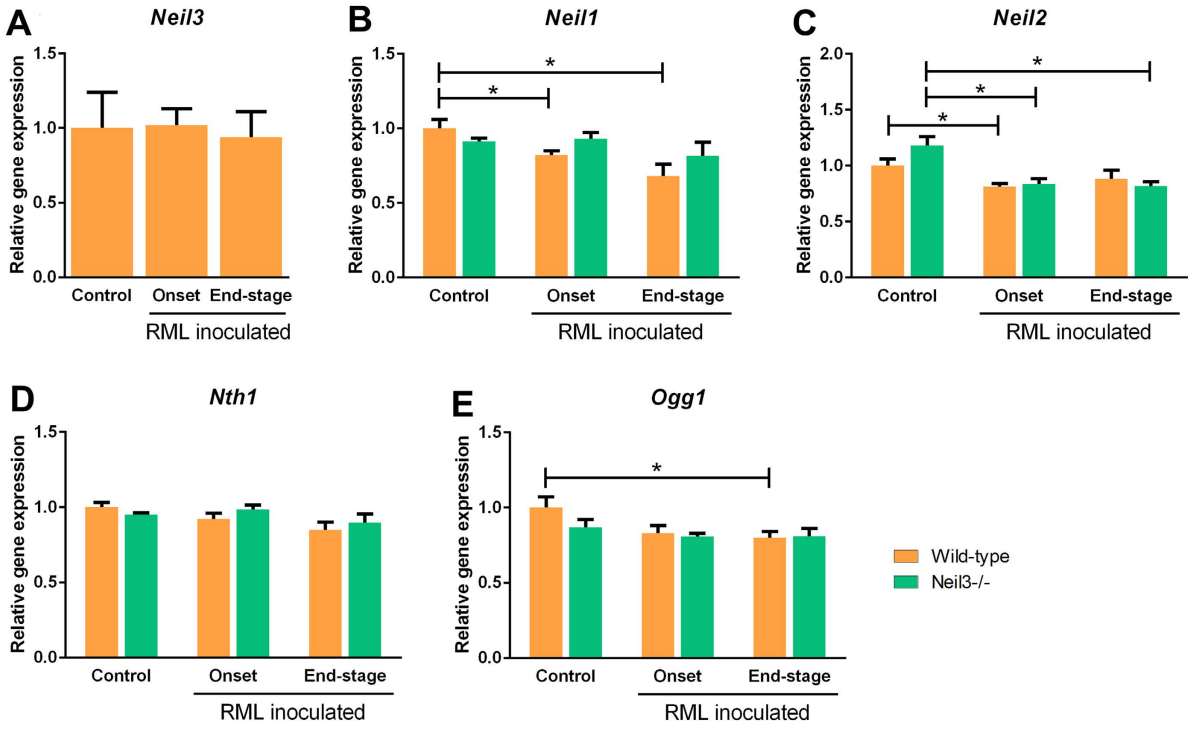

Figure 2. Expression of DNA glycosylases: nei endonuclease VIII-like, *Neil1*, *Neil2*, *Neil3*, DNA endonuclease III-like, *Nth1* and 8-oxoguanine DNA glycosylase, *Ogg1* in brain at onset and end-stage prion disease ($n = 4–6$, both groups), relative to expression in un-inoculated age-matched controls of wild-type mice ($n = 4–6$). Figure shows mean values with SEM.

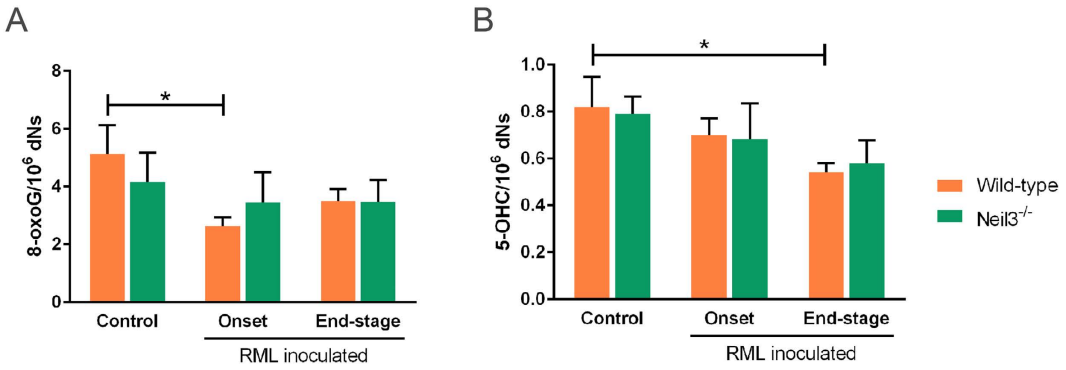

Figure 3. Mass spectrometry measurement of genomic 8-oxo guanine (8-oxoG) and 5-hydroxy cytosine (5-OHC) levels in whole brain of un-inoculated controls of wild-type ($n = 4–6$) and Neil3 KO ($n = 4–6$) mice and RML inoculated mice of both groups at onset ($n = 4–6$, both groups) and at end-stage ($n = 4–6$, both groups). Figure shows mean values with SEM.

proliferative cells in the subgranular layer, which increases as the disease progresses[10]. At the terminal stage, the neurogenic process is fading, as the Dcx+ cells in dentate gyrus are not observed.

Interestingly, we observed a mildly reduced astrogliosis in the Neil3 KO mice compared with the wild-type controls. This might reflect reduced proliferative capacity in the Neil3-deficient mice. Analysis of neuronal progenitor markers *Nestin*, *Sox2*, *Tuj1* and neuroblast marker *Dcx* revealed little difference between wildtype and Neil3 KO mice. All progenitor markers declined towards end-stage prion disease, except *Dcx* that was up-regulated in wild-type mice at end-stage, compared with onset of disease. This upregulation of Dcx was not evident in Neil3 deficient mice, supporting a role of Neil3 in activating neurogenesis during the clinical phase in response to neuronal loss. Expression levels of *NeuN*, a marker for mature neurons, dropped significantly more in Neil3-deficient brain than wild type during prion disease, probably due to severe neuronal loss, indicating that the neurogenic capacity is impaired in Neil3 KO. However, detailed morphometric and gene expression analysis using laser micro-dissected tissue samples or by *in situ* hybridization techniques are needed for comprehensive

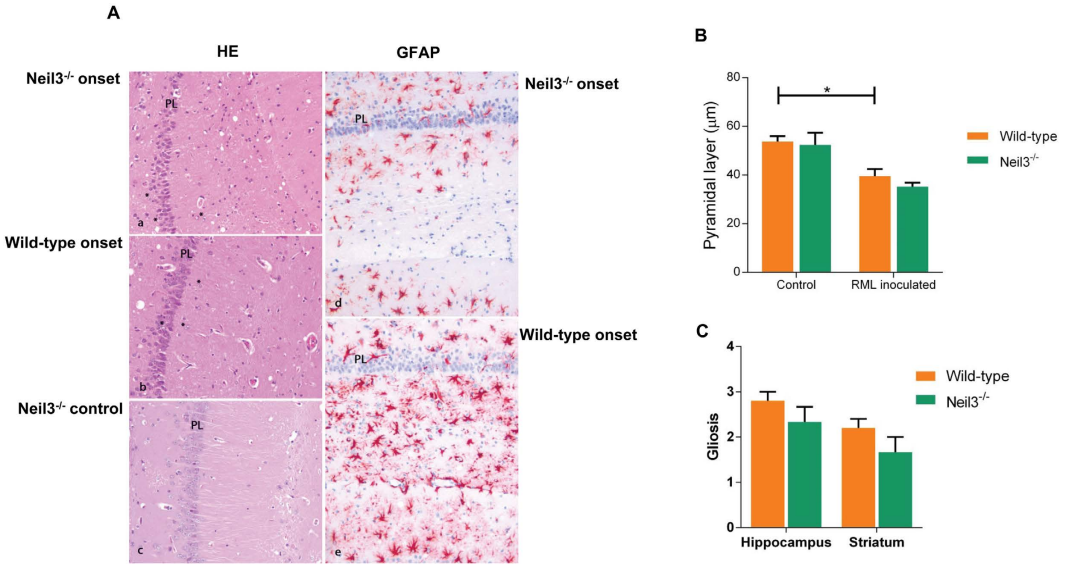

Figure 4. Histopathology (hematoxylin eosin) and astrogliosis (glial fibrillary acidic protein, GFAP) in hippocampus at onset of disease (**A**) in Neil3 KO and in wild-type mice inoculated with RML and in un-inoculated Neil3 KO control mice. The level of vacuolization is moderate and evenly distributed in both inoculated groups. Magnification: 200X. There was a reduction of the thickness of the pyramidal cell layer at onset of disease (**B**) in both groups, ($n = 4$ in each group). The astrocyte proliferation appeared more prominent in wild-type mice as compared to Neil3 KO mice in the hippocampus and in the striatum (**A**,**C**) ($n = 4$, both groups). Figure shows mean values with SEM (**B**,**C**).

assessment of neurogenesis at different stages of prion derived brain pathology in Neil 3 KO and in wild-type mice.

It has recently been demonstrated that activation of microglia contributes an over-all protective role against prion pathology, probably by phagocytosing harmful prion aggregates or cellular debris[28]. Our observation of shortened clinical duration in Neil3 KO mice could therefore be a result of compromised microglial activation in this genotype. In order to evaluate the activation state of microglia at onset and end-stage prion disease in wild-type and Neil3 Ko mice we compared expression levels of microglial markers *Cd68*, *Cd86* and inflammation markers *TNFα* and *Il1ß* at both time-points. As expected, already at onset of disease a marked microglial activation in both genotypes that increases even further towards the final stages of the disease is evident. Our data do not demonstrate major differences in microglial activation between wildtype and Neil3 KO mice, suggesting that loss of Neil3 does not compromise microglial activation and proliferation during prion-induced neurodegeneration.

Our observations of a significantly shortened clinical phase of prion disease in mice compromised in different BER enzyme activities, such as *Mutyh* and *Ogg1*[12] and here in *Neil3* deficient mice suggest that during clinical prion disease, DNA glycosylase activities provide neuroprotection. Since the physiological roles of these enzymes are diverse and partly unknown it is of considerable interest to elucidate underlying pathogenic commonalities that might reveal in molecular detail how DNA glycosylases prolong neuronal survival during the toxic phase of prion disease. The precise mechanisms by which DNA glycosylases contributes neuroprotection during prion disease awaits a deeper understanding of the physiological roles of these enzymes in the brain, beyond maintenance of genomic stability or canonical repair.

Materials and Methods

Ethics statement. This study was carried out in accordance with the Norwegian Regulation on Animal Experimentation, which is based upon the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and approved by the Norwegian Animal Research Authority, approval number 6232.

Mice. In these experiments, 36 female mice, 20 C57 Bl/6Y (wild-type) and 16 transgenic *Neil3*$^{-/-}$ mice, were inoculated intra-cerebrally with the RML isolate. The mice weighed between 18 and 22 grams at inoculation. Four mice were excluded from the experiment. Three mice, two wild-type and one *Neil3*$^{-/-}$, died within two days after inoculation due to complications and one was found dead 156 dpi, but material was of insufficient quality for further diagnosis and analysis. Four wild-type mice and three *Neil 3*$^{-/-}$ mice served as non-inoculated age-matched controls for the DNA-damage analysis. Wild-type C57 Bl/6 mice were bred in-house in the same room as *Neil 3*$^{-/-}$ mice. Mice were housed in groups of four in individually ventilated and sealed cages, under a 12-h light/ 12-h dark cycle at 21 °C, with food and water ad libitum. Animals were bred in accordance with European regulations FELASA category C. The genotypes of the mice were routinely tested by a PCR-based protocol.

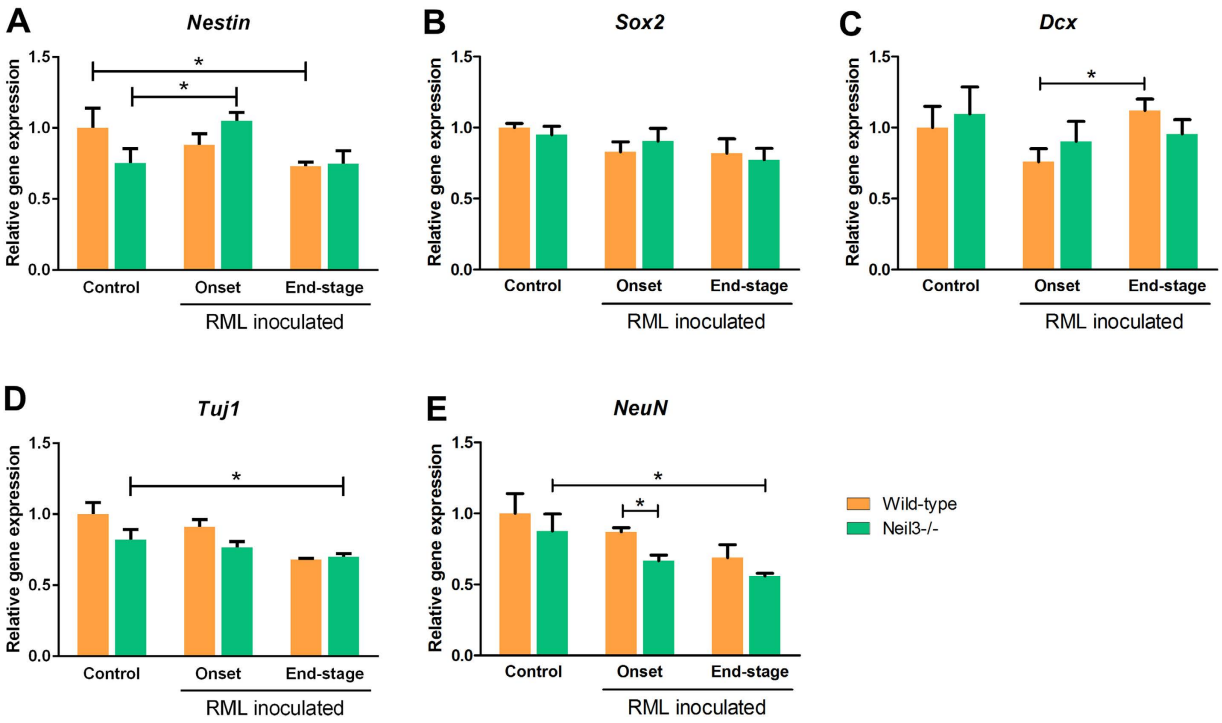

Figure 5. Whole brain expression levels of neuronal stem cell marker Nestin (*Nes*), Sex determining region Y box (*Sox2*) and immature neuron marker Doublecortin (*Dcx*), Class III beta-tubulin (*Tuj1*) and mature neuron marker Feminizing locus on X-3 (*NeuN*) in wild-type and Neil3 KO mice at onset and end-stage of prion disease, relative to age-matched un-inoculated controls. In both groups, as expected a significant drop in mature neuron marker *NeuN* was observed already at onset of disease and more prominent at end-stage. Expression levels of *Dcx* were mildly up-regulated at end-stage as compared to onset in both groups. $N = 4–6$ in all groups, data presented as means with SEM.

Inoculum. In this study, mouse adapted scrapie strain RML, which is originally derived from sheep scrapie brain homogenate (SSBP-1) was provided by Dr Andreoletti (ENV Toulouse, France). This prion strain has been passaged in many lines of mice for many decades and adapted into C57Bl/6Y. The mice were anesthetized with a mixture of tiletamine, zolezepam and xylazine that was injected subcutaneously, and 20 µl of a 10% inoculum was injected into the right midhemisphere.

Monitoring and sampling. The mice were monitored daily and signs of clinical disease, such as stiff tail, ataxia, hyperexcitability, ruffled coat, kyphosis, paraparesis, trembling and lethargy, were recorded.

In order to characterize the progression of disease parameters from onset to end-stage prion disease, 8 C57Bl/6Y (wild-type) and 8 transgenic *Neil3*$^{-/-}$ mice were euthanized at onset of early clinical signs. When the predefined clinical endpoint was reached, 9 C57Bl/6Y (wild-type) and 7 transgenic *Neil3*$^{-/-}$ mice were killed by dislocation of the craniocervical junction and sampled. The brain was cut longitudinally. One half of the brain was fixed in 10% neutral buffered formalin. After fixation, the brain tissue was cut into five transverse sections as described by Bruce and co-workers[29] and processed for histopathological analysis and immunohistochemical (IHC) detection of PrP^{Sc} and the astrocyte marker, glial fibrillary acidic protein (GFAP). The second half of the brain was frozen and later used for detection of PrP^{Sc} by western blot (WB).

For the DNA damage analysis, one-half of the brain and the spleen were sampled into RNAlater, to stabilize and protect cellular RNA. The second half was used for histo-pathological and IHC analysis.

Lesion scoring and immunohistochemistry. After fixation and processing, the brain tissue was cut and embedded in paraffin. Sections (4 µm thickness) were routinely deparaffinized, rehydrated and stained with hematoxylin and eosin (HE). The following brain areas were blindly scored for lesion profiling by two pathologists; dorsal half of the medulla oblongata, the cerebellar cortex of the folia, the cortex of the superior colliculus, hypothalamus, thalamus, hippocampus, the septal nuclei, cerebral cortex, dorsal to the corpus callosum and the cerebral cortex dorsal to the septal nuclei. The degree of vacuolization was scored from zero (no vacuoles) to five (dense vacuolization). The thickness of the hippocampal pyramidal layer was measured blindly using LAS camera software at six different points in each section and the mean was calculated. The intensity of GFAP labelling was semi-quantitatively scored according to the following criteria: 0, negative; 1, little; 2, moderate; 3, strong labelling.

For detection of PrP^{Sc}, the sections were pretreated by immersion in 98% formic acid for 30 min, washed and autoclaved at 121 °C in 0.01 M citric acid, pH 6.1 for 30 min, prior to incubation with proteinase K (4 µg/ ml) at 37 °C for 5 minutes. A cocktail of primary antibodies (F89/160.1.5 1/2000 and 2G11 1:400) was applied for 1 h at room temperature and for visualization of PrP^{Sc}, a commercially available kit (EnVision®+ System–HRP

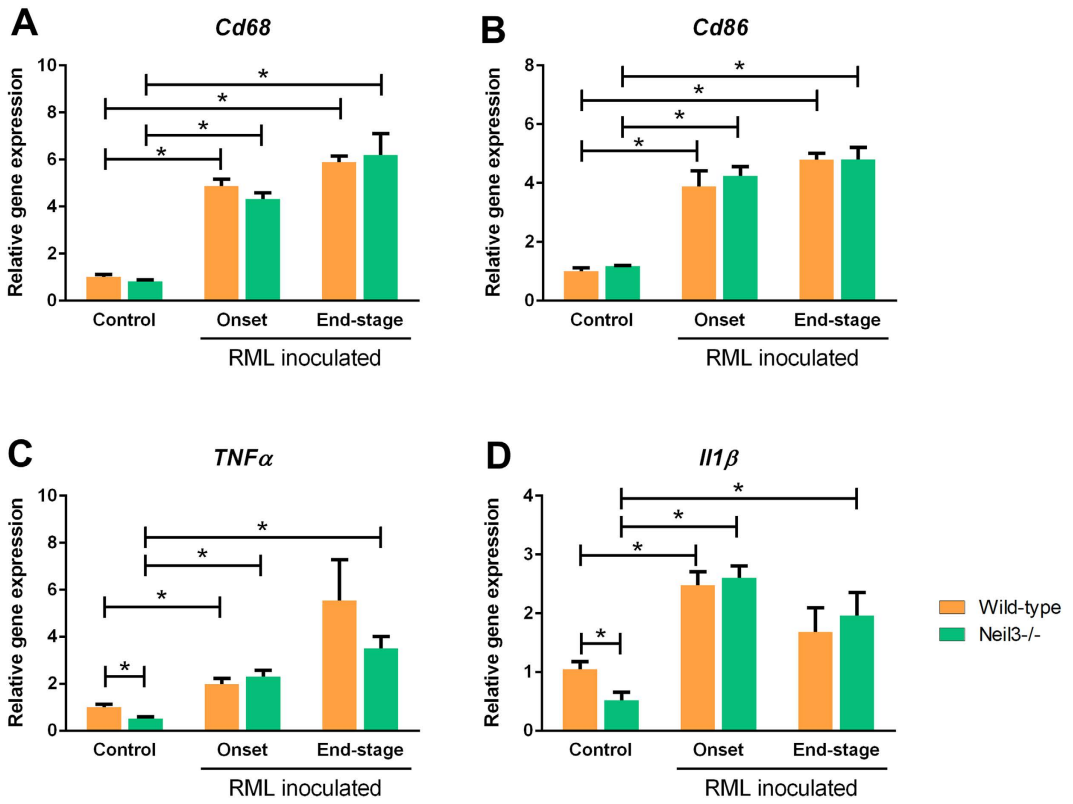

Figure 6. Whole brain expression levels of microglial gene markers, at onset and end-stage prion disease, relative to un-inoculated controls: Cluster of differentiation 68 (CD68) and 86 (CD86), tumor necrosis-factor alpha (TNFα) and interleukin 1-beta (Il1ß). N = 4–6 in all groups. There is a profound increase in all markers at onset of disease, which appears similar between controls and Neil3 KO mice. With the exception of Il1ß, which decreases slightly towards end-stage, all markers increase further towards end-stage prion disease.

(AEC) Mouse, DAKO) was used. Staining with GFAP was performed with a polyclonal rabbit anti-cow GFAP antibody (Dakopatts) at 1: 2000 dilution for 1 h at room temperature and detection by use of the kit EnVision®+ System-HRP (AEC) Rabbit (DAKO).

Western blot analysis for detection of PrP^{Sc}. Brain samples were homogenized at room temperature in a buffer containing Tris-HCl pH 7.4, 150 mM NaCl, 0.5% (w/v) Triton X-100, 0.5% sodium deoxycholate, and treated with Proteinase K (4 μg/ml) for 1 h at 37 °C, or with PNGase F (New England Biolabs) for 2 h at 37 °C for deglycosylation. Samples were boiled in SDS sample buffer and run on 12% pre-cast polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes by a semi-dry blotter. Membranes were blocked for 1 h in 5% fat-free dried milk, and incubated with primary antibody Bar224 (Spi Bio, France) overnight at 4 °C. Secondary antibody (goat anti-mouse) labeled with alkaline phosphatase (ALP: GE Healthcare, UK) was used to visualize bands with a fluorescence imager (Typhoon 9200, GE Healthcare, UK) after incubation with the ALP substrate (ECFTM, Western blotting reagent pack, GE Healthcare). In addition, an EU and OIE approved test, (TeSeE WESTERN BLOT, Bio-Rad) was used according to the recommendations of the manufacturers. The immunodetection was performed using the monoclonal antibody SHa31 that recognizes the amino acids (YEDRYYRE), corresponding to codons 145–152 in human PrP. Peroxidase activity was revealed using enhanced chemiluminescence (ECL, GE Healthcare, UK) substrate.

Transcription analysis of DNA glycosylases. Brain hemispheres were stored in RNAlater for at least 24 h before homogenization with FastPrep-24 (MP Biomedical). Total RNA was isolated using the Allprep DNA/RNA/Protein isolation kit (Qiagen) and 1 μg reversely transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene targets and corresponding primers for quantitative amplifications are given in Table 1. Relative expression levels were calculated using the comparative Δct method and related to the housekeeping gene *ß-actin*.

LC-MS/MS quantification of 8-oxodG and 5ohdC. DNA samples were digested by incubation with a mixture of nuclease P1 from *Penicillium citrinum* (Sigma, N8630), DNaseI (Roche, 04716728001) and ALP from *E. coli* (Sigma P5931) in 10 mM ammonium acetate buffer pH 5.3, 5 mM $MgCl_2$ and 1 mM $CaCl_2$ for 30 min at 40 °C. The samples were methanol precipitated, supernatants were vacuum centrifuged at room temperature until dry, and dissolved in 50 μl of water for LC/MS/MS analysis. Quantification was performed with the use of an LC-20AD HPLC system (Shimadzu) coupled to an API 5000 triple quadrupole (ABSciex) operating

Target	Forward	Reverse
Neil1	GCTGACCCTGAGCCAGAAGAT	CCCCAACTGGACCACTTCCT
Neil2	TTTAGTGGTGGTGGCTTCCT	TGATGTTCCCTAATCCTGAGAAG
Neil3	GGGCAACATCATCAAAAATGAA	CTGTTTGTCTGATAGTTGACACACCTT
Nth1	CCCGGAGCCGTTGCA	TGCTCTCCAGCCAGACCAA
Ogg1	GTGACTACGGCTGGCATCC	AGGCTTGGTTGGCGAAGG
Nestin	TCTCCAGAAGAGGAGGACCA	TTCGAGAGATTCGAGGGAGA
Sox2	TCCAAAAACTAATCACAACAATCG	GAAGTGCAATTGGGATGAAAA
Dcx	TACCTGGGATTTTCCTTTGG	CTCGTTCGTCAAAATGTCCA
Tuj-1	CCAAGACAAGCAGCATCTGT	CAGAGCCAAGTGGACTCACA
Rbfox3 (*NeuN*)	GAGTCTATGCCGCTGCTGAT	TTGCTAGTAGGGGGTGAAGC
Cd68	CCAATTCAGGGTGGAAGAAA	ATGGGTACCGTCACAACCTC
Cd86	CTCTTTCATTCCCGGATGGT	GGAGGGCCACAGTAACTGAA
Tnfα	ACGGCATGGATCTCAAAGAC	GTGGGTGAGGAGCACGTAGT
Il1β	CAGGCAGGCAGTATCACTCA	TGTCCTCATCCTGGAAGGTC

Table 1. Gene targets and corresponding primer sets used for quantitative amplification.

in positive electrospray ionization mode. The chromatographic separation was performed with the use of an Ascentis Express C18 2.7 μm 150 × 2.1 mm i.d. column protected with an Ascentis Express Cartridge Guard Column (Supelco Analytical) with an Exp Titanium Hybrid Ferrule (Optimize Technologies Inc.). The mobile phase consisted of A (water, 0.1% formic acid) and B (methanol, 0.1% formic acid) solutions. The following conditions were employed for chromatography: for unmodified nucleosides – 0.13 mL/min flow, starting at 10% B for 0.1 min, ramping to 60% B over 2.4 min and re-equilibrating with 10% B for 4.5 min; for 5-oh(dC) - 0.14 mL/min flow, starting at 5% B for 0.1 min, ramping to 70% B over 2.7 min and re-equilibrating with 5% B for 5.2 min; for dC modifications, and 8-oxo(dG) - 0.14 mL/min flow, starting at 5% B for 0.5 min, ramping to 45% B over 8 min and re-equilibrating with 5% B for 5.5 min. For mass spectrometry detection the multiple reaction monitoring (MRM) was implemented using the following mass transitions: 252.2/136.1 (dA), 228.2/112.1 (dC), 268.2/152.1 (dG), 243.2/127.0 (dT), 244.1/128 [5-oh(dC)], 284.1/168.1 [8-oxo(dG)].

Statistical analysis. Graphical presentations and statistical analyses were performed with the GraphPad Prism 6 software package. *P*-values < 0.05 were considered statistically significant. Kaplan-Meier survival plots were compared with Log Rank Mantel Cox test, whereas mean values of DNA damage, morphometric data and gene expression comparisons were analyzed by Student's *t*-test.

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Author Contributions

C.M.O.J., K.S., S.L.B., M.B. and M.A.T. designed the study, performed experiments, analyzed data and wrote the manuscript. G.G., T.M. and C.E. performed experiments and analyzed data. R.S. provided technical support, including mouse breeding and genetic analysis. All authors reviewed the manuscript.

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Paper III

DNA glycosylase Neil2 is protective during clinical prion disease.

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Abstract

The DNA glycosylase Neil2 is a member of the base excision repair, BER, family of enzymes, which are important for repair of oxidative DNA damage. Specifically, Neil2 participates in repair of oxidized bases in single-stranded DNA of transcriptionally active genes. Mice with genetic ablation of Neil2 (*Neil2*$^{-/-}$) display no overt phenotypes, but an age-dependent accumulation of oxidative DNA damage and increased inflammatory responsiveness. In young mice intra-cerebrally inoculated with prions, vigorous prion propagation starts rapidly in the germinal follicles of the spleen due to inoculum spillover. Here, we compare experimental prion disease in *Neil2*$^{-/-}$ mice with that in wild-type mice at disease onset and end-stage. Specifically, we investigated disease progression, accumulation of DNA damage, and mitochondrial respiratory complex activity in brain and spleen. We used genome-wide RNA sequencing of the spleen to compare the immune responses to prion propagation in the spleen between the two groups of mice, at both onset and end-stage prion disease. The *Neil2*$^{-/-}$ mice deteriorated more rapidly than wild-type mice after onset of clinical signs. Levels of DNA damage in brain increased in both mouse groups, slightly more in the *Neil2*$^{-/-}$ mice. Transcriptome data from spleen were similar between the mouse groups at disease onset with moderate genomic responses. However, at end-stage a substantial response was evident in the wild-type mice. Our data show that Neil2 counteracts toxic signaling in clinical prion disease, and this is separate from gross pathological manifestations and PrP^{Sc} accumulation.

Introduction

Prion diseases are invariably fatal, neurodegenerative maladies caused by disease-provoking protein complexes, known as prions. These consist of misfolded conformers of the host-encoded cellular prion protein PrP^{C} (Pan, Baldwin et al. 1993, Prusiner 1996). The pathogenic aggregates often display some protease resistance, and are known as PrP^{Sc} when revealed in immunohistochemistry or western blots. In some naturally occurring prion diseases, such as classical scrapie in sheep and chronic wasting disease in deer, PrP^{Sc} aggregates are often abundantly present in peripheral lymphoid tissues along the gastrointestinal tract and in the spleen long before onset of clinical signs (Hadlow, Kennedy et al. 1982, Lasmézas, Cesbron et al. 1996, Brown, Wathne et al. 2009).

In experimental prion disease in mice, induced by intracerebral inoculation with the mouse-adapted scrapie strain RML (Rocky Mountain Laboratory), a similar situation occurs, with vivid peripheral prion propagation in the spleen that is probably initiated by inoculum spillover

(Millson, Kimberlin et al. 1979). It has long been known that prions propagate in the spleen (Fraser and Dickinson 1970, Kimberlin and Walker 1988) and that peak infectivity levels are reached much faster in spleen than in brain (Rubenstein, Merz et al. 1991, Race, Ernst et al. 1992). The facility of prion propagation in the spleen is also of importance regarding cross species (Béringue, Herzog et al. 2012) transmission as well as strain developments (Béringue, Le Dur et al. 2008, Chapuis, Moudjou et al. 2016). The tissue fixed follicular dendritic cells, which are located in the white pulp germinal centers is of pivotal importance for prion propagation in the spleen (Jeffrey, McGovern et al. 2000, McCulloch, Brown et al. 2011) and PrP^{Sc} accumulations are mainly located in these follicles. In accordance with this, we have also observed active prion propagation in the spleen in RML-infected mice that are compromised regarding repair of oxidative DNA damage. Animals express a battery of DNA repair glycosylases with overlapping activities, especially in the base excision repair (BER) pathway (Krokan, Standal et al. 1997, Krokan and Bjørås 2013). Interestingly, genetic disruption of BER enzymes generally reveals no overt phenotypes related to genomic instability or cancer development. The full range of physiological roles of BER enzymes is incompletely understood and probably goes beyond canonical DNA repair and maintenance of genomic quality. However, gene knockout of downstream effectors, such as APE1 or pol ß are embryonic lethal, suggesting that accumulation of BER pathway intermediates is lethal (Xanthoudakis, Smeyne et al. 1996, Larsen, Gran et al. 2003, Puebla-Osorio, Lacey et al. 2006).

RML prion disease in mice with combined knock-out of the DNA repair enzymes 8-oxoguanin and mutY DNA glycosylase, *Ogg1*$^{-/-}$ and *Mutyh*$^{-/-}$, and in mice with disruption of the Nei endonuclease VIII-like 3 gene, *Neil3*$^{-/-}$, indicates that DNA glycosylases contribute to neuroprotection during the clinical stages of neurodegeneration without altering overall disease features (Jalland, Benestad et al. 2014, Jalland, Scheffler et al. 2016). Here we have used the same experimental paradigm to address prion disease development in mice that lack expression of the DNA glycosylase Neil2 (*Neil2*$^{-/-}$). Since these mice have been shown to be hyper-responsive to certain forms of inflammatory insults (Chakraborty, Wakamiya et al. 2015), we were particularly interested in studying prion disease progression in the spleen, in addition to brain pathology.

Materials and Methods

Ethics statement: All animal work was carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other

Scientific Purposes and according to Norwegian Regulations of Animal Experimentation (FOTS). Approval number 6232.

Mice: In these experiments, 34 female mice, 20 C57Bl/6Y (wild-type) and 14 transgenic *Neil2*$^{-/-}$ mice, were inoculated intra-cerebrally with the RML isolate. All mice were female and weighed between 18 and 22 grams at inoculation. Two wild-type mice that died two days after inoculation were excluded from the experiment. One wild-type mouse was found dead 156dpi, but material was of insufficient quality for further diagnosis and analysis. Four wild-type mice and three *Neil2*$^{-/-}$ mice served as non-inoculated age-matched controls for the DNA-damage analysis. Wild-type C57Bl/6 mice were bred in-house in the same room as *Neil2*$^{-/-}$ mice. Mice were maintained in high grade facility under a 12-h light/12-h dark cycle at 21°C, with food and water ad libitum, and housed in groups of four in individually ventilated and sealed cages. Breeding of the animals was done in accordance with European regulations FELASA category C. The genotypes of the mice were assessed by a PCR-based protocol.

Inoculum: Mice were inoculated with the mouse adapted scrapie strain RML, which is originally derived from sheep scrapie brain homogenate (SSBP-1), and has been passaged in many lines of mice for many decades and adapted into C57Bl/6Y. The anesthesia was preformed with a mixture of tiletamine, zolezepam and xylazine, which was injected subcutaneously. Mice were intracerebrally inoculated, into the right midhemisphere, with a 20 µl of a 10% inoculum.

Monitoring and sampling: The animals were monitored daily. Signs of clinical disease, such as stiff tail, ataxia, ruffled coat, hyperexcitability, kyphosis, hind leg paresis, trembling, and lethargy, were recorded.

In order to characterize the progression of disease, animals were sacrificed at onset and end-stage of disease. In total, 15 animals, 9 wild-type and 6 transgenic *Neil2*$^{-/-}$ mice were euthanized at onset of early clinical signs. At the predefined clinical endpoint, 8 wild-type and 8 transgenic *Neil2*$^{-/-}$ mice were sacrificed and the material was collected. The brain was cut in two halves. One half was fixed in 10% neutral buffered formalin, and later used for immunohistochemistry (IHC). The other half was frozen and later used for detection of PrP^{Sc} by western blot (WB).

For DNA damage analysis, one-half of the brain and the spleen were sampled into RNAlater, to stabilize and protect cellular RNA. The second half was used for histopathological and IHC analysis.

Lesion scoring and immunohistochemistry: After fixation, the brain tissue was cut into five transverse sections (Bruce and Dickinson 1982) and embedded in paraffin. Sections (4 µm thickness) were routinely stained with haematoxylin and eosin (HE) and blindly scored for lesion profiling by two pathologists; dorsal half of the medulla oblongata, the cerebellar cortex of the folia, the cortex of the superior colliculus, hypothalamus, thalamus, hippocampus, the septal nuclei, cerebral cortex, dorsal to the corpus callosum and the cerebral cortex dorsal to the septal nuclei. The vacuolization was scored from zero (no vacuoles) to five (dense vacuolization). The thickness of the hippocampal pyramidal layer was measured blindly using LAS camera software at six different points in each section and the mean was calculated.

For histological detection of PrP^{Sc}, the sections were pretreated by immersion in 98% formic acid for 30 min, washed and autoclaved at 121 °C in 0.01 M citric acid, pH 6.1 for 30 min, prior to incubation with proteinase K (4 µg/ ml) at 37 °C for 5 minutes. The primary antibodies (F89/160.1.5 1/2000 and 2G11 1:400) was applied for 1 h at room temperature. To detect PrP^{Sc}, a commercially available kit (EnVision®+ System–HRP (AEC) Mouse, DAKO) was used. The staining with GFAP, for astrocytes, was performed with a polyclonal rabbit anti cow GFAP antibody (Dakopatts) at 1: 2000 dilution for 1 h at room temperature and detection by use of the kit EnVision®+ System-HRP (AEC) Rabbit (DAKO).

Pet Blot: Brain and spleen tissue was cut (3-5 µm) from paraffin imbedded tissue and placed on 0.45 µm nitrocellulose membrane, with glass slide underneath, and dried for approximately 1 day. The membranes were deparaffinized, rehydrated and placed in buffer with 250 µg/ml Proteinase K (Sigma-Aldrich) on top of towel paper, pillow technique, over night at 55 °C. The membranes were washed with Tris-buffered saline with 0.1% Tween 20, before denaturation in 4M guanidiumthiocynate for 15 minutes and washed 3 x 5 minutes with TBST. Membranes were blocked in 0.2% casein in PBS with 0.1% Tween 20 for 45 minutes, and incubated with primary antibody, Sha31, for 90 minutes at room temperature. Secondary antibody, goat anti-mouse labeled with alkaline phosphatase, in TBST was applied for 60 minutes. The signals were visualized through Formazan reaction with NBT/BCIP. The staining process was stopped by incubation of the sections in PBS for 15-30 minutes. To achieve a long-lasting staining the membranes were incubated in ddH_2O and 0.5M EDTA for 1-2 hours.

Western blot analysis for detection of PrP^{Sc}: To detect PrP^{Sc}, the brain samples were homogenized at room temperature in a buffer containing Tris-HCl pH 7.4, 150 mM NaCl,

0.5% (w/v) Triton X-100, 0.5% sodium deoxycholate, and treated with Proteinase K (4 µg/ml) for 1 h at 37 °C, or with PNGase F (New England Biolabs) for 2 h at 37 °C for deglycosylation. PK digestion was stopped by adding SDS sample buffer and boiling the samples at 95 °C for 5 minutes. Proteins were separated on 12% pre-cast polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes by a semi-dry blotter. Membranes were blocked in 5% fat-free dry milk for 1 h, before incubation with primary antibody, Bar224 (Spi Bio, France), over night at 4 °C. Secondary antibody (goat anti-mouse) labeled with alkaline phosphatase (ALP: GE Healthcare, UK) was applied to visualize bands with a fluorescence imager (Typhoon 9200, GE Healthcare, UK) after incubation with the ALP substrate (ECFTM, Western blotting reagent pack, GE Healthcare). According to the recommendations of the manufacturers, an EU and OIE approved test, (TeSeE WESTERN BLOT, Bio-Rad) was used. The immunodetection was performed using the monoclonal antibody SHa31 that recognizes the amino acids (YEDRYYRE), corresponding to codons 145–152 in human PrP. Peroxidase activity was revealed using enhanced chemiluminescence (ECL, GE Healthcare, UK) substrate.

Mitochondrial complex activity analyses: Mitochondria from freshly prepared tissue samples were isolated and complex I and V activity measured as described previously (Halsne, Esbensen et al. 2012).

Nucleic acid extraction: Tissue samples were stored in RNAlater for at least 24 h before homogenization with FastPrep-24 (MP Biomedical). Genomic DNA and total RNA were isolated using the Allprep DNA/RNA/Protein isolation kit (Qiagen).

RT-qPCR: cDNA was synthesized from 1 µg total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression of DNA glycosylases were measured using the following primers: *Neil1*, forward: 5' GCTGACCCTGAGCCAGAAGAT 3' and reverse: 5' CCCCAACTGGACCACTTCCT 3', *Neil2*, forward: 5' TTTAGTGGTGGTGGCTTCCT 3' and reverse: 5' TGATGTTCCCTAATCCTGAGAAG 3', *Neil3* forward: 5' GGGCAACATCATCAAAAATGAA 3' and reverse: 5' CTGTTTGTCTGATAGTTGACACACCTT 3', *Nth1*, forward: 5'CCCGGAGCCGTTGCA 3' and reverse: 5' TGCTCTCCAGCCAGACCAA 3', *Ogg1*, forward: 5'GTGACTACGGCTGGCATCC 3' and reverse: 5' AGGCTTGGTTGGCGAAGG 3'. Relative expression levels were calculated using the comparative Δct method and related to the housekeeping gene *ß-actin*.

RNA sequencing: Total RNA from spleen of wild-type (n = 4-6) and *Neil2*$^{-/-}$ mice (n= 4) from control, onset, and end-stage were pooled and sent to BGI Tech Solutions, Hong Kong, for RNA sequencing and bioinformatics analysis. Differentially expressed genes (DEGs) were filtered using log2Ratio ≥ 1 and false discovery rate (FDR) ≤ 0.001 as cut off. Comparison of DEGs was performed by using Venny 1.0 online tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Ingenuity pathway analysis (IPA) software was used to identify enriched pathways and upstream regulator of DEGs.

Mitochondrial DNA damage detection: Real-time qPCR was used to quantify mitochondrial DNA damage as described in (Wang, Scheffler et al. 2016). Briefly, genomic DNA was digested with Taq$^{\alpha}$I restriction enzyme and subsequent real-time PCR was carried out to amplify non-damaged DNA with site-specific mitochondrial DNA primer. Relative amounts of PCR products were calculated by the comparative ΔC_T method. The following primers were used: *mt-Rnr1*, forward: 5′-ACTCAAAGGACTTGGCGGTA and reverse, 5′-AGCCCATTTCTTCCCATTTC.

LC-MS/MS quantification of 8-oxodG and 5ohdC: DNA samples were digested by incubation with a mixture of nuclease P1 from *Penicillium citrinum* (Sigma, N8630), DNaseI (Roche, 04716728001) and ALP from *E. coli* (Sigma P5931) in 10 mM ammonium acetate buffer pH 5.3, 5 mM $MgCl_2$ and 1 mM $CaCl_2$ for 30 min at 40ºC. The samples were methanol precipitated, supernatants were vacuum centrifuged at room temperature until dry and dissolved in 50 µl of water for LC/MS/MS analysis. Quantification was performed with an LC-20AD HPLC system (Shimadzu) coupled to an API 5000 triple quadrupole (ABSciex) operating in positive electrospray ionization mode. The chromatographic separation was performed with the use of an Ascentis Express C18 2.7 µm 150 x 2.1 mm i.d. column protected with an Ascentis Express Cartridge Guard Column (Supelco Analytical) with an Exp Titanium Hybrid Ferrule (Optimize Technologies Inc.). The mobile phase consisted of A (water, 0.1% formic acid) and B (methanol, 0.1% formic acid) solutions. The following conditions were employed for chromatography: for unmodified nucleosides – 0.13 mL/min flow, starting at 10% B for 0.1 min, ramping to 60% B over 2.4 min and re-equilibrating with 10% B for 4.5 min; for 5-oh(dC) - 0.14 mL/min flow, starting at 5% B for 0.1 min, ramping to 70% B over 2.7 min and re-equilibrating with 5% B for 5.2 min; for dC modifications, and 8-oxo(dG) - 0.14 mL/min flow, starting at 5% B for 0.5 min, ramping to 45% B over 8 min and re-equilibrating with 5% B for 5.5 min. For mass spectrometry detection the multiple reaction monitoring (MRM) was

implemented using the following mass transitions: 252.2/136.1 (dA), 228.2/112.1 (dC), 268.2/152.1 (dG), 243.2/127.0 (dT), 244.1/128 [5-oh(dC)], 284.1/168.1 [8-oxo(dG)].

Statistical analysis: Graphical presentations and statistical analyses were performed with the GraphPad Prism 6 software package. *P*-values < 0.05 were considered statistically significant. Kaplan-Meier survival plots were compared with Log Rank Mantel Cox test, whereas mean values of DNA damage, morphometric data and gene expression comparisons were analyzed by Student's *t*-test.

The p-values, in RNA sequencing, were calculated using Fisher's exact test.

Results

Pre-inoculation steady state levels of brain PrP^{C} glycoforms and major processing fragment C1 were similar between the mouse genotypes (Figure 1A). At onset and end-stage prion disease both mouse groups accumulated similar levels of PrP^{Sc}, and the glycoforms were indistinguishable (Figure 1B). Differences in the brain pathology disease profile at onset (Figure 1C) and end-stage (Figure 1D) in the mouse groups were small. However, the trend was, that in the hypothalamus (brain area 4), the pathological alterations were less severe at onset in the *Neil2*$^{-/-}$ mice, and that these mice at end-stage were slightly less affected in the hippocampus (area 6) and the cerebral cortex (area 8). Although the first clinical signs appeared simultaneously in the two genotype mouse groups, the *Neil2*$^{-/-}$ mice had faster disease progression and were sacrificed according to human end-point relatively synchronously. In the wild-type mice, however, disease progressed more slowly and some animals had still not been sacrificed more than 20 days after onset of clinical signs (Figure 1E). In accordance with a more protracted clinical disease, the wild-type mice suffered greater reduction in body mass from onset to end-stage of disease, with a 24% mean reduction in body mass, whereas the *Neil2*$^{-/-}$ dropped 11% of their body mass from onset to end-stage (Figure 1 F). As shown in Table 1, the loss of Neil2 activity did not result in upregulation of other DNA glycosylases in the spleen. At onset and end-stage prion disease there was a tendency towards a drop in expression levels of DNA glycosylases, which, in the case of Neil3, was significant in both genotype groups.

Analysis of genome-wide accumulation of oxidized base lesions in brain showed no difference between *Neil2*$^{-/-}$ and wild-type mice (Figure 2A). In spleen, there were significantly lower levels of 5ohdC in both groups of mice, but most prominent in Neil2 deficient mice. Importantly, 5ohdC is a specific substrate for Neil2. At end-stage, levels were similar between the two groups of mice (Figure 2 B). Neil2 has been suggested to have in role in maintenance of the

mitochondrial genome (Mandal, Hegde et al. 2012). In addition, mitochondrial dysfunction has been observed in brain of scrapie-infected mice (Park, Kim et al. 2011, Choi, Choi et al. 2014). Surprisingly, we found no difference in mitochondrial DNA (mtDNA) damage level in brain between the mouse genotypes, although mtDNA damage accumulated significantly during the course of disease. In contrast, *Neil2*$^{-/-}$ showed significantly ($p < 0.05$) less mtDNA damage in spleen at end-stage than was found in wild-type (Figure 3A).

Enzymatic activity of complex I in isolated mitochondria from brain was stable during the course of disease, whereas complex V activity increased only in wild-type brain at end-stage compared with onset (Figure 3B). Interestingly, mitochondrial respiratory activity in *Neil2*$^{-/-}$ spleen was significantly increased at end-stage, both for complex I and complex V, whereas in wild-type mice this apparent upregulation was absent (Figure 3C).

Transcriptome changes were evident at onset of the disease, with 373 and 384 DEGs in wild-type and *Neil2*$^{-/-}$ spleens respectively, as compared with un-inoculated controls. At end-stage, wild-type mice dramatically increased in DEGs, with 582 upregulated and 1379 downregulated genes, in contrast to *Neil2*$^{-/-}$ mice that had minor differences in number of DEGs at end-stage compared with onset (Figure 4A). In addition, *Neil2*$^{-/-}$ DEGs overlapped almost 60% with wild-type DEGs at end-stage, suggesting a lack of transcriptional response in *Neil2*$^{-/-}$ spleen at end-stage of prion disease (Figure 4A, B).

The top five significantly affected pathways included cell cycle, proliferation, and DNA replication and repair processes (Figure 4C). Interestingly, DEGs from *Neil2*$^{-/-}$ spleen at end-stage were enriched in proliferation of immune cells, such as leukocytes and lymphocytes (Figure 4D). In addition, upstream mediators involved in cell cycle control and proliferation processes were commonly activated at end-stage, indicating enhanced proliferation of immune cells in *Neil2*$^{-/-}$ spleen. However, wild-type mice showed, in contrast, inhibition of proliferation during the course of disease, demonstrated by a negative z-score of these upstream regulators (Figure 4E).

Moreover, the transcriptome data demonstrated that genes involved in mitochondrial function were significantly upregulated in *Neil2*$^{-/-}$ spleen at end-stage. We found DEGs contributing both to complex I and complex V activity and several regulators of mitochondrial transcription and translation, including transcription factor B1, mitochondrial translational initiation factor 2 and several mitochondrial ribosomal proteins. These genes, in contrast, were downregulated in wild-type spleen, comparing un-inoculated controls with end-stage mice (Figure 4F).

There were no gross pathological differences in the spleen between *Neil2*$^{-/-}$ mice and wild-type and the overall splenic architecture appeared intact (Figure 5). At end-stage, amyloid masses were evident in the center of splenic follicles and this appeared slightly more pronounced in the *Neil2*$^{-/-}$ mice (Figure 5 panels B and E), but limitations in material prevented quantification. Paraffin embedded tissue blots (PET) demonstrated prominent follicular PrP^{Sc} deposits that appeared similar between the genotypes (Figure 5 panels C and F).

Levels of 5mC significantly dropped at onset of disease in wild-type and *Neil2*$^{-/-}$ spleen. However, wild-type mice maintained similar levels of 5mC at end-stage compared with onset, whereas at end-stage in *Neil2*$^{-/-}$ mice 5mC levels returned back to the level of un-inoculated controls (Figure 6 A). In comparison, 5hmC slightly increased during disease progression in wild-type spleen, but not in *Neil2*$^{-/-}$ spleen, resulting in significantly lower level of 5hmC at onset and end-stage (Figure 6B). The effects on epigenetic modifications were specific for spleen; brain levels of 5mC and 5hmC were stable during the disease and similar between the genotypes (Suppl. Figure 1).

Discussion

As has previously been observed in experimental prion disease in mice with compromised DNA glycosylase activity (Jalland, Benestad et al. 2014, Jalland, Scheffler et al. 2016), the overall features of prion disease were similar in *Neil2*$^{-/-}$ mice and wild-type mice. At the onset of disease, however, there was a tendency towards milder effects in *Neil2*$^{-/-}$ mice, with a lower disease score in the hypothalamus and somewhat lower levels of oxidative DNA damage, particularly in the spleen. Nevertheless, the *Neil2*$^{-/-}$ mice deteriorated very rapidly once clinical signs were evident and the synchronicity of these mice in reaching the clinical end-points suggested that they had reached a critical threshold, incompatible with neuronal survival. Interestingly, this occurred with such rapidity that the animals lost much less of their body mass than seen in the wild-type mice, in which the protracted clinical course included many days of anorexia.

Whereas mitochondrial functions, expressed as respiratory complex activity, remained unaffected in wild-type mice throughout the disease course, both in brain and spleen, the *Neil2*$^{-/-}$ mice appeared to mount a burst of mitochondrial activity in the spleen at end-stage. This was not evident in brain, in which activities were indistinguishable from wild-type mice. Spleen transcriptional responses also revealed a proliferative response in the *Neil2*$^{-/-}$ mice compared with the wild-type mice. Increased proliferation of cells could contribute to the observed increase in mitochondrial activity. The difference between the genotypes could in this regard,

be influenced to some extent by the profound anorexia observed in the wild-type mice, which could initiate anorexia-mediated atrophy of the spleen. The proliferative response in the spleen could also contribute to potentially harmful pro-inflammatory signaling that could in turn, contribute to the rapid clinical deterioration of these animals.

Considering that the *Neil2*$^{-/-}$ mice have been shown to be in an induced state of innate immunity signaling and hyper-reactive towards inflammatory stimuli such as LPS and TNF-alpha (Chakraborty, Wakamiya et al. 2015) it is tempting to speculate that the loss of Neil2 accelerates toxic prion disease signaling and thereby leads to rapid death. However, the spleen morphology and follicular accumulation of PrP^{Sc} was quite similar between the genotype groups, with accumulations of what appeared to be amyloid masses in germinal centers. It has previously been reported that prion propagation (ME7 strain) in spleen can lead to disruption of the splenic follicular architecture (Kim, Han et al. 2016), but this was not a prominent feature our model, in which the general architecture of the spleen appeared intact even at end-stage prion disease.

Epigenetic DNA modifications regulate gene transcription and Neil2 has recently been found to cooperate with thymine DNA glycosylase (TDG) during oxidative DNA demethylation (Schomacher, Han et al. 2016). To evaluate possible effects on the epigenome in spleen of *Neil2*$^{-/-}$ mice that contribute to transcriptional alterations at end-stage, we measured the level of 5mC and 5hmC in genomic DNA. We observed no major differences in global 5ohc level in brain and spleen in Neil2-deficient mice. However, Neil2 is known to preferentially repair oxidized DNA bases from single-stranded DNA (Dou, Mitra et al. 2003) and transcriptionally active genes (Chakraborty, Wakamiya et al. 2015). Thus, we cannot exclude that gene-specific accumulation of oxidative lesions occur that contribute to the observed phenotype. In line with its role in transcribed regions of the genome, we found diminished transcriptional response in *Neil2*$^{-/-}$ spleen during the course of disease suggesting that Neil2 is important for regulation of gene expression. Interestingly, levels of 5mC changed significantly during prion disease with increased levels in *Neil2*$^{-/-}$ spleen at end-stage. Given the importance of epigenetic marks for gene regulation, it is tempting to speculate that Neil2 functions in epigenetic remodeling in the context of prion disease. Recent findings demonstrating a role for Neil2 in the process of active demethylation support this idea (Schomacher, Han et al. 2016).

Taken together, our data suggest that distinct from detectable morphological alterations, and levels and distribution of PrP^{Sc} deposits, Neil2 activity counteracts toxic signaling in prion disease. This is in accordance with data demonstrating that Neil2, in contrast to OGG1, promotes anti-inflammatory signals.

348

349

Figure 1. Pre-inoculation levels of PrP^C, glycoforms, C1 fragment and major disease characteristics in wild-type and *Neil2*$^{-/-}$ mice. A) Steady-state levels of brain PrP^C levels, glycoforms and proteolytic processing were similar between the genotypes. B) Western Blot of brain PrP^{Sc} at onset and end-stage. Lesion distribution at C) onset (n = 8 wt, 7 *Neil2*$^{-/-}$) and D) end-stage (n = 9 wt, 6 *Neil2*$^{-/-}$) in nine defined brain areas (1 medulla oblongata, 2 cerebellar cortex, 3 cortex of the superior colliculus, 4 hypothalamus, 5 thalamus, 6 hippocampus, 7 septal nuclei, 8 cerebral cortex dorsal to corpus callosum, 9 cerebral cortex dorsal to septal nuclei). E) Survival plot of wild-type (n = 9) and *Neil2*$^{-/-}$ (n = 6) mice after inoculation with RML prions. F) Body mass of wild-type and *Neil2*$^{-/-}$ (means +/- SEM, n = 6-11 per group) mice at onset and end-stage of prion disease. ** ($p < 0.01$), * ($p < 0.05$), Student's t-test

Figure 2. Quantification of global oxidative DNA base lesions in brain and spleen at onset and end-stage prion disease in wild-type and *Neil2*$^{-/-}$ mice. Mass spectrometry-based detection of genomic 8-oxoG and 5ohC level of un-inoculated and RML-inoculated wild-type and *Neil2*$^{-/-}$ mice at onset and end-stage of disease in A) brain and B) liver**.** Data presented as means (n = 3-6 per group and time-point), +/- SEM. * ($p < 0.05$).

Figure 3. Mitochondrial burst in spleen of *Neil2*$^{-/-}$ mice at end-stage of prion disease. Mitochondrial function was analyzed in brain and spleen of un-inoculated (n = 4) and RML-inoculated wild-type and *Neil2*$^{-/-}$ mice at onset (n = 4-5) and end-stage (n = 4-6) of disease. A) Quantitative PCR-based analysis of mitochondrial DNA damage in brain and spleen. B) Assessment of brain and C) spleen mitochondrial complex I and complex V activity in controls and RML-inoculated wild-type and *Neil2*$^{-/-}$ mice.

Figure 4. RNA sequencing reveals enhanced proliferation of immune cells and increased expression of mitochondrial genes in spleen of *Neil2*$^{-/-}$ mice at end-stage of prion disease. A) Differentially expressed genes (DEGs) in wild-type (n = 4-6) and *Neil2*$^{-/-}$ (n = 4) spleen at onset and end-stage of prion disease, as compared with un-inoculated controls. B) Venn diagram showing overlap of DEGs at end-stage compared with controls from wild-type and *Neil2*$^{-/-}$ mice. Ingenuity pathway analysis showing C) Top five biological processes and D) biological processes involving proliferation enriched DEGs from *Neil2*$^{-/-}$ compared with wild-type mice at end-stage of prion disease. E) Activation score of top five upstream regulators enriched in DEGs from wild-type mice at end-stage compared with controls and DEGs from end-stage *Neil2*$^{-/-}$ mice compared with wild-type. F) Heatmap showing log2 fold change of DEGs involved in mitochondrial function from wild-type mice at end-stage compared to controls and end-stage *Neil2*$^{-/-}$ mice compared to wild-type.

Figure 5. Morphology of spleen lymphoid follicles and PrP^{Sc} accumulations at onset and end-stage prion disease. Hematoxylin eosin stained tissue sections of spleen lymphoid follicles from wild-type (panels A and B) and *Neil2*$^{-/-}$ (panels D and E) mice at onset and end-stage prion disease (magnification 40x). PrP^{Sc} follicular accumulation in spleen was visualized by paraffin-embedded tissue blots (Panels C and F), magnification 10x.

Figure 6. *Neil2*$^{-/-}$ mice show increased DNA methylation levels in spleen at end-stage of prion disease. Mass spectrometry-based detection of genomic 5mC and 5hmC in spleen of un-inoculated and RML-inoculated wild-type and *Neil2*$^{-/-}$ mice at onset and end-stage of disease. Data presented as means (n = 3-6 per group and time-point), +/- SEM. * ($p < 0.05$).

Supplementary Figure 1. No differences in genomic levels of epigenetic modifications in brain. Mass spectrometry-based detection of genomic 5mC and 5hmC in brain of un-inoculated and RML inoculated wild-type and *Neil2*$^{-/-}$ mice at onset and end-stage of disease. Data presented as means (n = 4-6 per group and time-point), +/- SEM. * ($p < 0.05$).

Table 1 Relative gene expression of DNA glycosylases in spleen

Gene	Wild-type			Neil2$^{-/-}$		
	Control	Onset	End-stage	Control	Onset	End-stage
Neil1	1,00 ± 0.20	1,24 ± 0.12	0,69 ± 0.15[b]	1,109 ± 0.13	0,960 ± 0.04	0,778 ± 0.09
Neil2	1,00 ± 0.17	0,76 ± 0.09	1,46 ± 0.32	-	-	-
Neil3	1,00 ± 0.25	0,31 ± 0.04[a]	0,18 ± 0.04[a]	0,764 ± 0.15	0,277 ± 0.05[a]	0,232 ± 0.07[a]
Nth1	1,00 ± 0.18	0,88 ± 0.07	1,00 ± 0.09	0,891 ± 0.10	0,743 ± 0.05	0,823 ± 0.04
Ogg1	1,00 ± 0.08	1,00 ± 0.07	0,93 ± 0.02	0,931 ± 0.09	0,810 ± 0.05	0,828 ± 0.05[c]

p<0.05 vs control [a], vs onset [b] and vs wt [c]

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Author Contributions Statement

KS, CMOJ, SLB, MB and MAT designed the study, performed experiments, analyzed data and wrote the manuscript. GG, TM and CE performed experiments and analyzed data. RS provided technical support, including mouse breeding and genetic analysis. All authors reviewed the manuscript.

Additional Information

The authors declare no competing financial interests.

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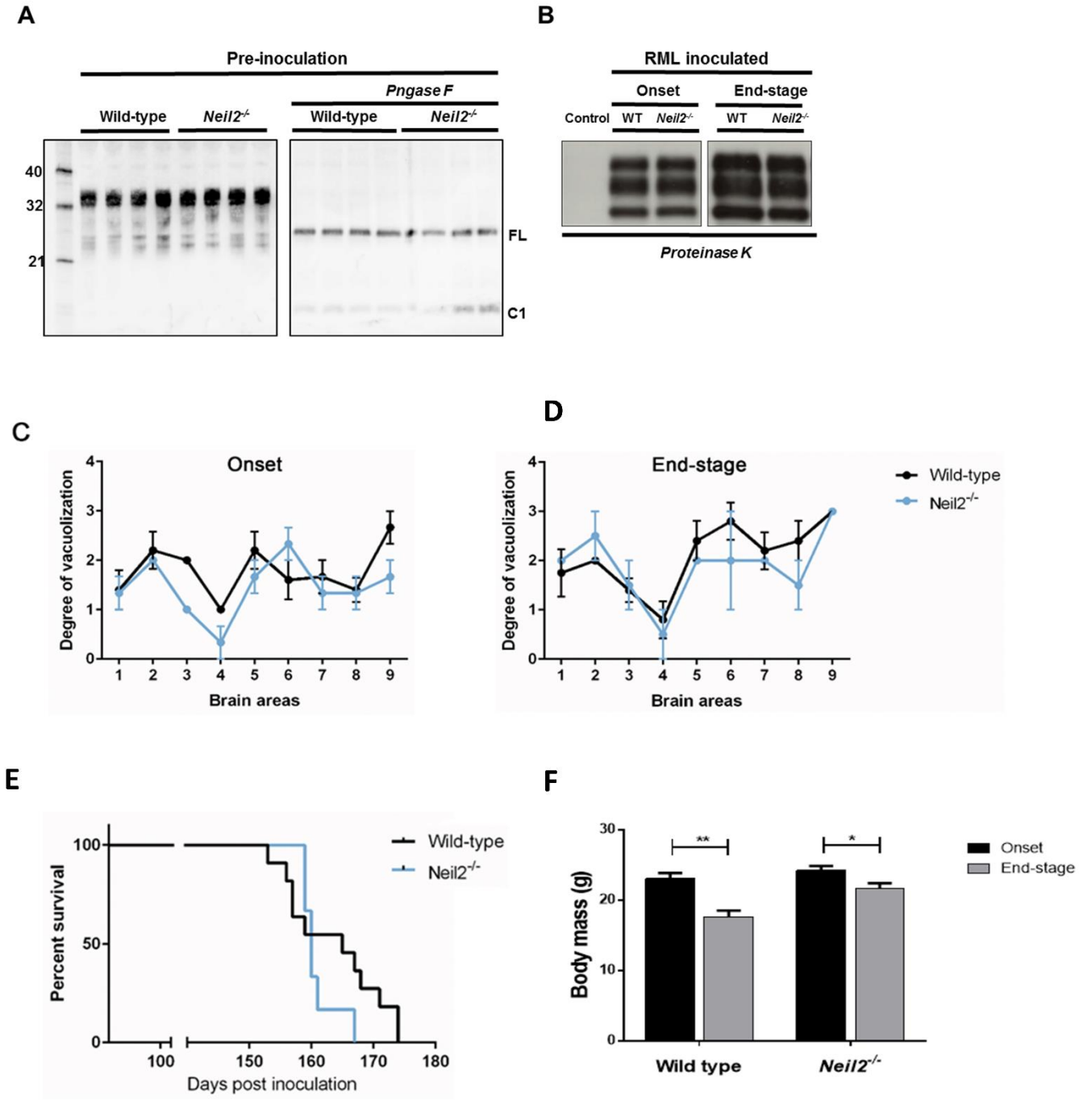
Figure 1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Pngase F
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Neil2-/-
Wild-type
Neil2-/-
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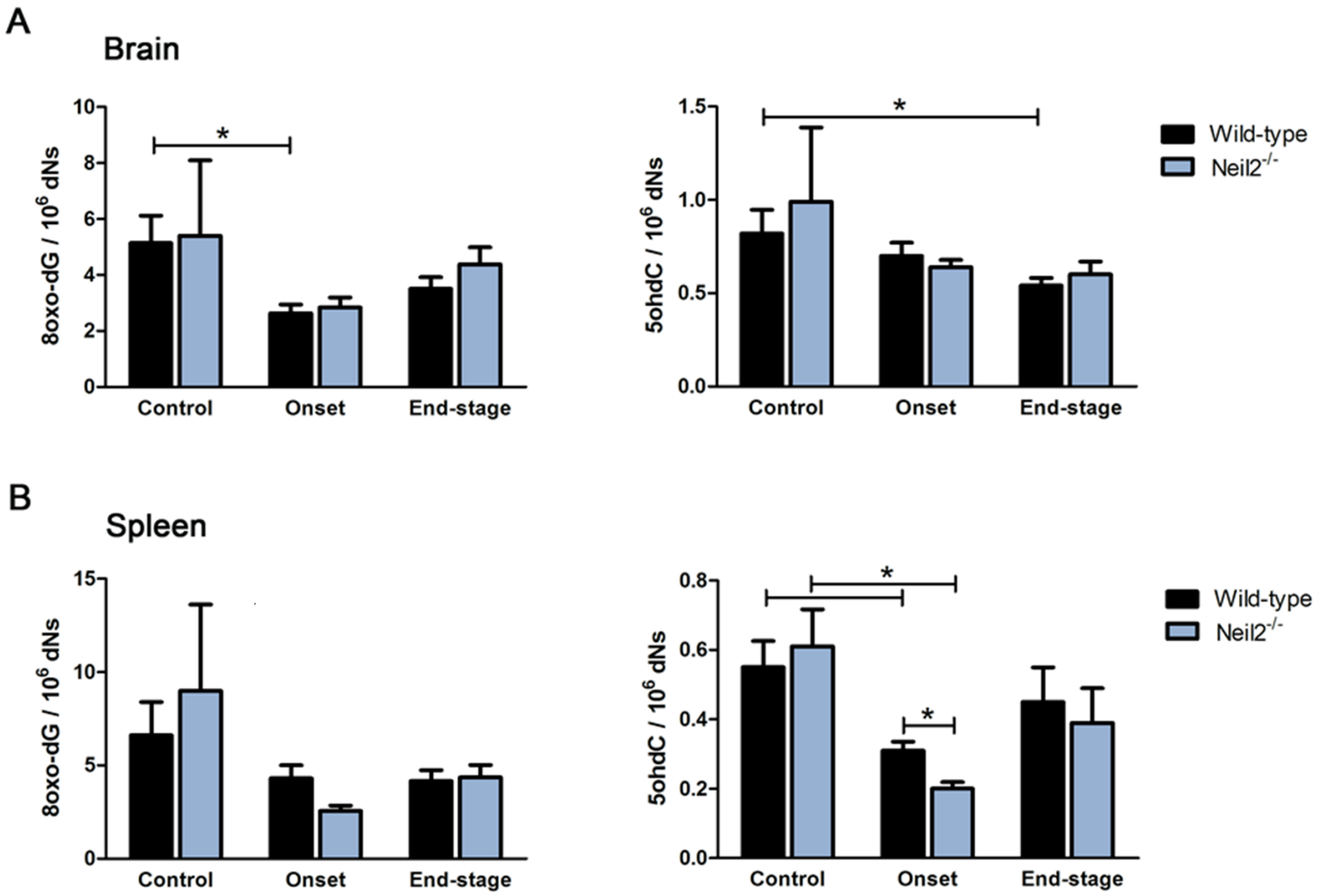
32
21
FL
C1
B
RML inoculated
Onset
End-stage
Control
WT
Neil2-/-
WT
Neil2-/-
Proteinase K
C
Onset
Degree of vacuolization
Brain areas
D
End-stage
Degree of vacuolization
Brain areas
Wild-type
Neil2-/-
E
Percent survival
Days post inoculation
Wild-type
Neil2-/-
F
Body mass (g)
**
*
Onset
End-stage
Wild type
Neil2-/-

Figure 2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Brain
8oxo-dG / 10^6 dNs
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8
6
4
2
0
*
Control
Onset
End-stage
5ohdC / 10^6 dNs
1.5
1.0
0.5
0.0
*
Control
Onset
End-stage
Wild-type
Neil2-/-
B
Spleen
8oxo-dG / 10^6 dNs
15
10
5
0
Control
Onset
End-stage
5ohdC / 10^6 dNs
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Control
Onset
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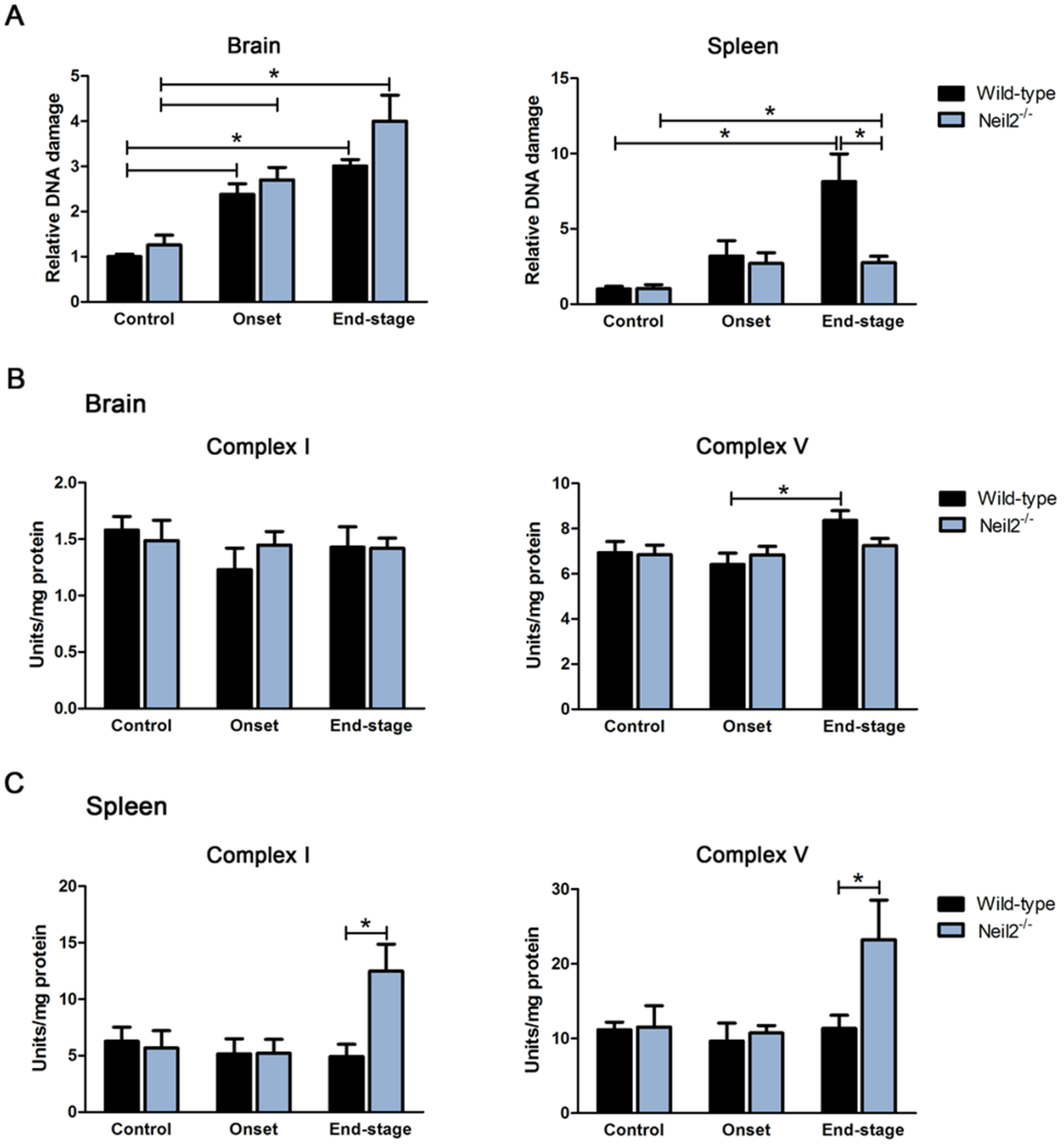
Wild-type
Neil2-/-

Figure 3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Brain
Relative DNA damage
Control
Onset
End-stage
Spleen
Wild-type
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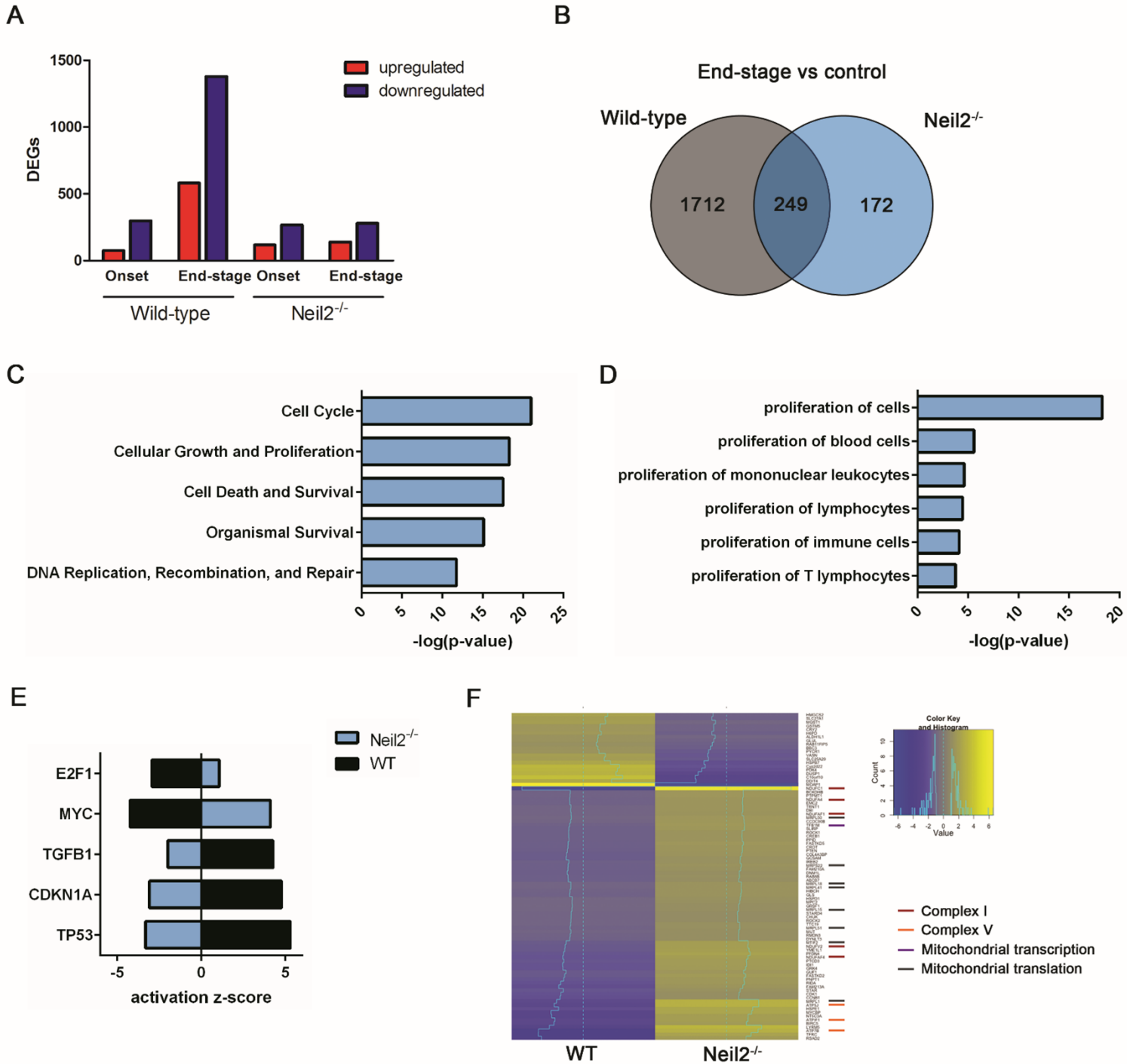
Units/mg protein
Complex V
C
Spleen

Figure 4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1000
500
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DEGs
upregulated
downregulated
Onset
End-stage
Onset
End-stage
Wild-type
Neil2-/-
B
End-stage vs control
Wild-type
Neil2-/-
1712
249
172
C
Cell Cycle
Cellular Growth and Proliferation
Cell Death and Survival
Organismal Survival
DNA Replication, Recombination, and Repair
0
5
10
15
20
25
-log(p-value)
D
proliferation of cells
proliferation of blood cells
proliferation of mononuclear leukocytes
proliferation of lymphocytes
proliferation of immune cells
proliferation of T lymphocytes
0
5
10
15
20
-log(p-value)
E
Neil2-/-
WT
E2F1
MYC
TGFB1
CDKN1A
TP53
-5
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Complex V
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Mitochondrial translation
WT
Neil2-/-

Figure 5

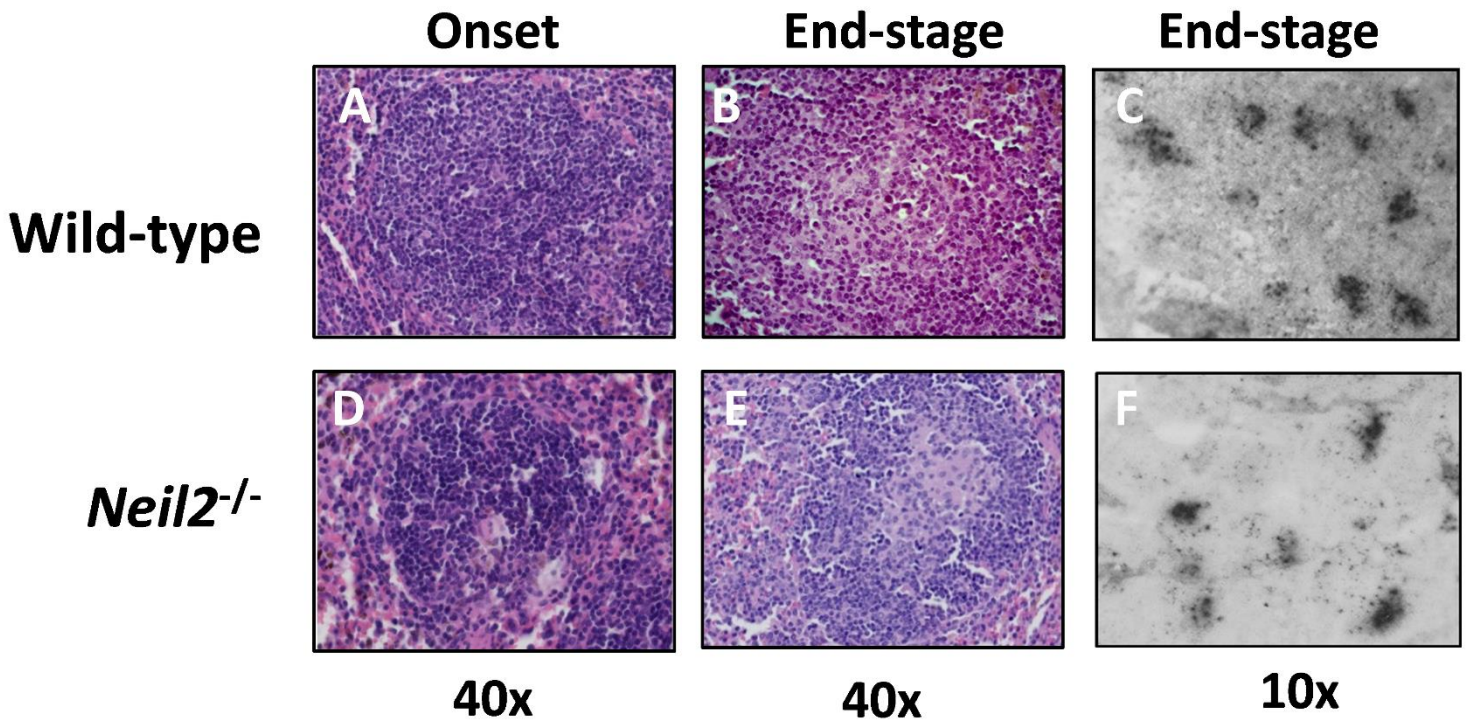
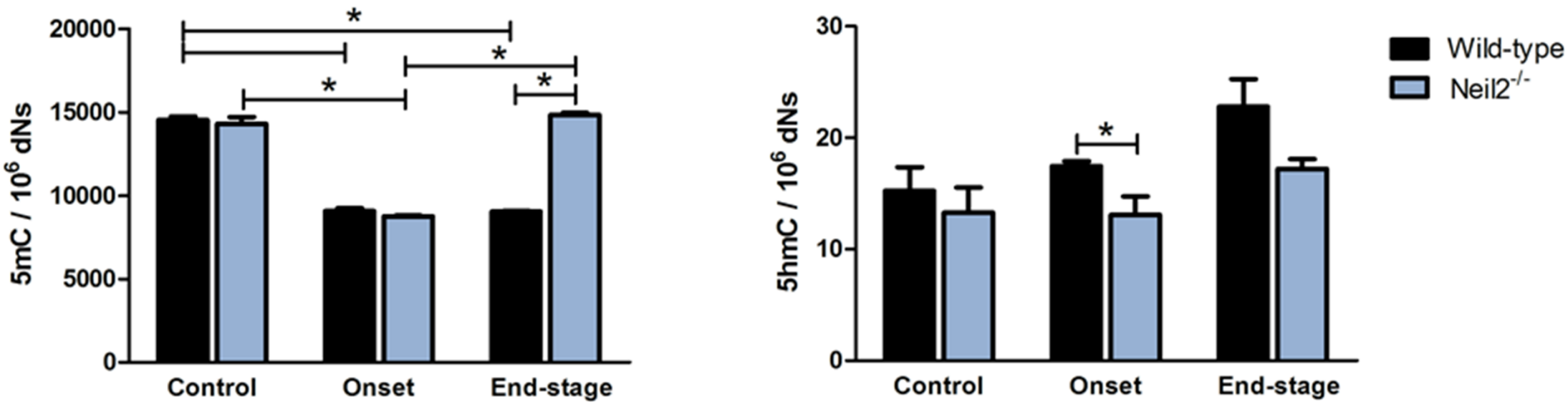


Figure 6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*
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*
*
5hmC / 10^6 dNs
30
20
10
0
Control
Onset
End-stage
*
Wild-type
Neil2-/-

Supplementary figure 1