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Time-course study of retinal pathology in mice infected with scrapie

by

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ABSTRACT

Infectious prions are proteinaceous pathogens that cause transmissible spongiform encephalopathies (TSEs). There is a specific nomenclature used in prion literature; the general notation for the prion proteins (infectious or non-infectious) is notated by PrP, while PrP^{Sc} and PrP^C are used to differentiate between the disease-associated and normal cellular forms, respectively.

TSEs are caused by an interaction between a misfolded and protease-resistant prion protein, PrP^{Sc} and its normal cellular counterpart, PrP^C. PrP^C is a cell-surface protein found throughout the nervous system. This interaction causes the normal isoform to refold into PrP^{Sc}, which then accumulates within neurons and glia causing widespread neuronal degeneration within the brain. Furthermore, PrP^{Sc} accumulation can be found in numerous other tissues, including the retina and lymphatic tissues. Retinal accumulation of PrP^{Sc} is known to occur with many TSEs, including scrapie in sheep and goats, and may prove to be valuable for non-invasive, antemortem diagnostics. The retina, as an extension of the diencephalon, could potentially serve as a window into concurrent pathology occurring in the central nervous system in TSEs. The purpose of this study was to investigate the time course of PrP^{Sc} accumulation, and resulting retinal changes in a mouse model of scrapie.

C57Bl/6 mice were inoculated with mouse adapted scrapie (RML-scrapie) brain homogenate, either intracerebrally or intraperitoneally. Animals were euthanized and tissues collected approximately every 30 days throughout the disease course, then prepared for standard histopathologic and immunohistochemical analysis. Immunohistochemistry was done

on retinal tissues using antibodies against PrP (monoclonal antibody 6C2) and Glial Fibrillary Acidic Protein (GFAP), which is upregulated under conditions of retinal stress. Cell counts of the outer nuclear layer were done to quantify retinal degeneration. We found retinal PrP^{Sc} accumulation, retinal stress, and retinal degeneration were detectable after approximately 80% of the incubation period in mice, regardless of inoculation route. Thus we now have a model for more detailed study of PrP^{Sc} accumulation and neuronal cell death.

The following paragraphs briefly discuss the history of prion diseases as well as an introductory description of the pathology. The discussion will review previous literature of retinal pathology associated with prion diseases, I will provide rationale for a study of scrapie-associated pathology in the retina over time.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

One of the oldest examples of prion disease was described in the Fore tribe in Papua New Guinea. The Fore had established a ritual that involved consuming the remains of deceased members of the tribe. This led to transmission of what the tribe referred to as “kuru”, a degenerative, progressive neurologic condition. This disease was more commonly seen in women, as after the men in the tribe had eaten what were considered the most substantial organs, the women were left with those deemed less important, which happened to include the brain and nervous system [1-4].

In the 1950’s the pathogenesis, lesions, and clinical signs of kuru were noted to share numerous similarities to an affliction of sheep, commonly known as scrapie. Most notably in its comparison to kuru, scrapie (so called due to the nature of affected animals to waste away, and “scrape” away their own wool) produced similar spongiform lesions in brain tissues [1-4]. Ovine genetic predispositions to scrapie infection have been linked to certain polymorphisms in the prion protein at codons 136, 154, and 171 [5,6]. Infected sheep display neurodegenerative symptoms including but not limited to: self-isolation, circling, anorexia/weight loss, and an unkempt hair coat [5]. They also demonstrate self-mutilating behavior by rubbing against objects and breaking off clumps of wool, effectively scraping off their own hair coat- lending the disease its name. As was seen with kuru, once individuals begin to show symptoms of scrapie, they continue to steadily and irreversibly decline.

Spongiform lesions in the brain had also been described in another human disorder, Creutzfeldt-Jakob disease (CJD), along with other rare neurodegenerative diseases such as Gerstmann-Straussler-Scheinker syndrome and fatal familial insomnia [7]. Like scrapie and kuru, these diseases were invariably progressive and ultimately fatal yet the root cause was long unknown [7]. However, it became apparent that the etiologic agent was unlike any pathogen that had been documented previously.

Stanley Prusiner, documented a number of distinctions through his work with scrapie. In 1982 he asserted the infectious particles were proteinaceous in nature, rather than viral or bacterial [8]. This was evidenced by failure to deactivate the infectious agent through use of methods specific for destroying nucleic acids, decreased infectivity following exposure to proteases, a heterogeneous nature, and small size of the infectious particles [8]. This new class of pathogen, unique in its ability to replicate without nucleic acids, was deemed a “prion” (derived from “infectious protein”).

With the emergence of a new spongiform encephalopathy, variant Creutzfeldt-Jakob disease (vCJD), in the 1980's identification and further study of the causative agent became imperative. vCJD produced similar lesions to sporadic CJD, however CJD was considered a slowly progressing neurodegenerative disease that developed later in life. In contrast, vCJD instead affected healthy young adults and progressed much more rapidly (2 years or less) [9,10]. Eventually, the origin of vCJD was traced to yet another neurodegenerative disorder: bovine spongiform encephalopathy (BSE) that was seen in cattle fed post-rendering scrap material, i.e. the meat and bone meal of other cattle [11]. The transmissibility of BSE to primates was confirmed in studies using lemurs and macaques inoculated with infectious brain

homogenate [12-14]. This was done in part by passage of BSE through mice and then inoculating non-human primates. The resulting strain of BSE was then found to quickly adapt to primates and exhibited very similar lesions and pathology to those seen in patients with vCJD [14].

As time went on, the similarities between scrapie, BSE, vCJD, CJD, and other spongiform encephalopathies could not be ignored, and so these diseases were grouped together and classified as transmissible spongiform encephalopathies (TSEs). Additionally, prions became more and more widely accepted as the root cause of TSEs.

This prion has been shown to be a misfolded isoform of a protein found in normal neurons (and other cells). The exact function of the cellular isoform remains unknown, however it has been linked to roles in reduction of oxidative stress, potentiating long-term memories, and cell adhesion/signaling [15]. While the abnormal prion protein, (PrP^{Sc}) and its normal cellular isoform (PrP^C) share identical amino acid sequences, their folded configurations differ in shape [16]. This difference in shape is also associated with different biophysical and chemical properties. PrP^{Sc} is more resistant to denaturation by heat, acid, and proteases than its cellular counterpart (PrP^C) [17]. The lower energy form is that of the infectious prion, PrP^{Sc} and as such PrP^C is induced to rearrange and refold itself into the infectious prion form when the two come into contact⁶⁹. This results in a chain reaction as more and more altered prion proteins exist and come into contact with more and more of their normal cellular counterparts. Infectious prions then accumulate intracellularly within the Golgi apparatus before extending into extracellular space as fibrils¹¹. Over time, this results in vacuolar neuronal degeneration and neuronal

apoptosis. This the family of disease caused by aberrant prion protein replication has come to be known as transmissible spongiform encephalopathies (TSEs) [2-3,9-10,8].

Prion pathogenicity has be further confirmed after isolation from infected animals. For example, homogenate from animals infected with scrapie inoculated into naïve animals has been shown to produce disease [8]. Furthermore, knockout animals that do not express cellular prion proteins have proven unsusceptible to TSE infection[8].

Thesis organization

This thesis provides an introduction to diseases caused by prions, and the characteristic properties of both the infectious prion protein and its normal cellular isoform. The following chapter provides a review of the existing literature on various inoculation routes used for experimentally generating TSE pathology, prion-associated retinal pathology, and TSE pathology studies using a time-course study design. Chapter 3 provides a manuscript describing a study of scrapie associated retinal pathology; materials and methodology are described, along with results and discussion sections, with figures following at the end. Chapter 4 gives a summary of findings, recommendations for future research, and concluding remarks.

CHAPTER 2: LITERATURE REVIEW

TSE research

As research into the nature of diseases caused by prions progressed, both time and cost constraints necessitated development of an appropriate model species for study. Selection of an appropriate model was complicated by both the long incubation periods seen with most TSEs, and a relative host specificity. In natural hosts, prion diseases develop gradually over the course of months to years. Scrapie most often occurs in 1-5 year old sheep, and BSE most often manifests in 4-6 year old cattle though the incubation period can range from 2-18 years [5]. Additionally, with the exception of BSE and vCJD, there are few cases in which a prion protein found in one species has been shown to be naturally infectious in another species.

These barriers were overcome through development of reliable methods of passing scrapie through rodents. The significantly shorter lifespan of hamsters (and later mice) proved immensely beneficial to studies of prion-related pathology over time. In contrast to larger animals with incubation periods of months to years, mice incubation periods tend to last 200-300 days or less [18]. Studies in mice also confer an economical benefit, especially when compared to research performed with primates, as was done in studies to support the infectivity of spongiform encephalopathies to humans [12-14].

Maira Bruce and her colleagues furthered research in using mouse models for passage of TSEs by devising a scoring system, to assist with identifying strains of prion diseases. This scoring system grades vacuolar degeneration (on a scale from 1-5) in 9 different gray matter sections: the dorsal medulla, cerebellar cortex, superior colliculus, hypothalamus, medial

thalamus, hippocampus, septum, medial cerebral cortex at the level of the thalamus, and the medial cerebral cortex at the level of the septum. Additionally, 3 white matter sections within the cerebellum, mesencephalic tegmentum, and pyramidal tract are also observed. These scores have been used to create a characteristic lesion profile for many different “strains” of prion disease, which exhibit different patterns of cerebral and cerebellar vacuolation [19-22]. These lesion profiles have also been shown to differ between routes of infection [23].

Different routes of inoculation

A wide variety of inoculation routes have been used in studying TSEs. These include intracerebral (IC), intraperitoneal (IP), and intraocular (IO), among others. While intracerebral inoculations generally produce disease more rapidly, they do not necessarily mimic the disease course as it occurs naturally. Naturally, infectious prion diseases are thought to be commonly acquired by ingestion, rather than direct inoculation into the CNS. A discussion of the differences seen with routes of inoculation follows, arranged from most to least effective at producing disease.

Intracerebral (IC) inoculations

Intracerebral inoculations have largely been associated with the most significant pathology [18,24]. In time-course studies, mice inoculated with mouse-adapted scrapie have reliably produced lesions more quickly than those seen with more peripheral routes (e.g. IP) [18,25]. In scrapie-infected mice, IC inoculations have produced lesions 110 days sooner than IP inoculations, and show a much shorter overall disease course (with death occurring around 170

days post inoculation for the IC route and at approximately 280 days post inoculation for the IP route) [18]. Similar results were seen in BSE infected mice [18]. Lesions in IC-inoculated mice were also found to be more severe, though they often followed similar distributions to those seen with the more peripheral routes [18]. Similarly, hamsters inoculated intracerebrally with 263K scrapie developed disease significantly more rapidly than their counterparts that were inoculated via other routes (e.g. IC, IP, IO, as well as intracardiac and intramuscular inoculations) [24].

Intralingual inoculations

An intralingual route of inoculation has also been used in comparison to IC using scrapie in its natural ovine host. The pathology and progression of disease seen with the two routes were very similar, which may be in part attributed to the heavy innervation of the tongue by cranial nerves 9 and 12, which provide direct access to the brainstem and CNS [26].

Intraperitoneal inoculations

This route of infectious prion injection is moderately effective at producing disease. However as mentioned above, though it can show a similar distribution to the intracerebral route, it is often less pronounced and development of significant lesions is delayed [18,24,27]. Furthermore, prion accumulation was somewhat decreased for mice inoculated with scrapie and BSE when brains from IP inoculated animals were compared to IC inoculated animals [18]. This delay of prion accumulation and decreased overall accumulation in IP inoculated animals

(when compared to IC inoculated animals) is not limited to the CNS, and has also been noted in lymphatic tissue [25].

Intraocular inoculations

Many studies that have used intraocular (IO) inoculations did not compare directly with other routes. However, to summarize, these studies did produce significant disease in the retina [28,29-30]. IO inoculation has also been shown to produce more disease in the inner retinal layers than extraocular inoculations [31]. This route has also been shown to produce CNS disease by anterograde transport via the optic nerve [32]. Though when CNS disease is produced it is not as severe as that seen with direct IC inoculation [24].

Oral and intragastric routes

Interestingly, the oral and intragastric routes would be most synonymous with what is thought to be the natural route of infection: ingestion. However, intragastric inoculation of scrapie into hamsters was not proven effective experimentally in causing disease [24]. Oral inoculation of CWD into cervids also has been shown to be ineffective in reliably producing disease except at high doses [33-34].

However, while a variety of inoculation routes have used experimentally, there is little to no literature comparing the pathological and immunohistochemical differences seen in the retinas using different inoculation protocols.

TSEs in the retina

In addition to their characteristic lesions in the cerebrum and cerebellum, TSEs have been documented as causing retinal pathology in numerous examples [35-38]. Retinal pathology correlates with disease in the rest of the central nervous system as the retina is actually a highly specialized extension of the diencephalon [39]. Thus, exploring retinal pathology could provide a peripheral “window” into the disease process occurring simultaneously in the brain.

Histologically the neural retina is divided into layers; from outermost to innermost these consist of the photoreceptor layer (PL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), the retinal ganglion cell layer (GCL), and the optic fiber layer (OFL). The photoreceptor layer can also be referred to as the outer segments (OS) which includes the connecting cilia and inner segments. The cell bodies of the photoreceptors make up the outer nuclear layer. The outer plexiform layer consists of bipolar cells processes, horizontal cells, and photoreceptor cells. The inner nuclear layer contains a variety of different cell body types, including bipolar cell bodies, amacrine cell bodies, horizontal cell bodies, and the cell bodies of Müller glia. Axons and synapses between these cells exist in the inner plexiform layer, which then contacts the ganglion cells of the ganglion cell layer. The optic fiber layer, which is in closest proximity to the vitreous body, contains unmyelinated axons of the ganglion cells. These axons converge centrally at the optic disc, and combine to form the optic nerve as they continue to the visual pathways of the brain [40].

While Müller glial cell bodies reside in the inner nuclear layer, these cells actually span the entire width of the retina, from photoreceptors to the optic fiber layer. Müller glia serve as

support cells for the retina, providing mechanical support, serving as glycogen stores, and regulating potassium levels in the retina. They also remove debris and provide a source of insulation to other cell types [40,41].

Under increased stress, glial cells (including Müller glia) increase expression of a cytoskeletal protein known as glial fibrillary acidic protein (GFAP) [41]. GFAP is only observed in endfeet of Müller glia under normal conditions, but when retinal pathology exists GFAP is expressed in significant amounts throughout Müller glial cells. This becomes important when pathology is in early stages, when few to no other histopathologic lesions are present. GFAP expression often occurs prior to noticeable retinal degeneration and can be an early indicator of disease processes. In cases of marked retinal degeneration and disease, GFAP expression increases and can also help to classify stage of disease. For these reasons, GFAP is often assessed in retinal studies by using immunohistochemistry, and has been commonly utilized in documentation of retinal pathology in TSEs [41].

Prion accumulation has been documented to occur in the retinas of infected animals, and GFAP expression has also been shown to increase as prions accumulate in the retina with TSEs [35,42]. In the following sections, descriptions of retinal pathology induced by infection with scrapie are discussed, focusing on scrapie in natural hosts, and experimental scrapie in mice.

Scrapie in sheep (and goat) retinas

As mentioned previously, scrapie naturally infects sheep (and more rarely goats); the disease most often occurs in sheep aged 1-5 years [5].

A simple, non-invasive route for ocular and fundic evaluation is direct or indirect ophthalmoscopy. However, though retinal pathology is known to occur few changes are seen on fundoscopic analysis. Sheep infected with scrapie cannot be reliably differentiated from non-infected animals via fundic examination with an ophthalmoscope. Furthermore, infected sheep did not display detectable cranial nerve deficits associated with optic tracts prior to showing clinical signs of disease [43]. Thus scrapie would be difficult to impossible to detect by routine ocular examination methods and more complex analyses are needed.

Another method for pre-mortem detection is electroretinography (ERG). This detects the electrical response of neurons in the eye following photic stimulation in dark surroundings. Electroretinography (ERG) on naturally infected sheep has shown decreased function when compared to controls, as indicated in this case by a lowered amplitude of responses [43]. Altered electroretinography (ERG) results have also been shown in a sheep experimentally inoculated with scrapie [44]. However, an altered ERG only shows that the retina is not functioning normally and is not necessarily specific for a TSE. Currently, the most effective methods for demonstrating the presence of prions in ocular tissues involve collection of tissues for histopathologic analysis.

Histopathologic analysis of retinal tissues has been used to document retinal degeneration in scrapie infected animals [42-43,45]. Disarray of the photoreceptor layer has been described, though not consistently in all literature [43]. However, multiple studies have reported thinning of retinal nuclear layers (both inner and outer) along with thinned outer plexiform layers [43,45].

Immunohistochemistry (IHC) can be used on fixed tissues as well to further classify histopathologic lesions. In retinas harvested from sheep inoculated with the agent of scrapie, GFAP has been demonstrated to increase in immunoreactivity in hypertrophied Müller glia cells [45]. Increased GFAP immunoreactivity has also been documented in the retinal ganglion cell layer and inner plexiform layer [43-44]. This increase occurs prior to detectable retinal degeneration [44].

Importantly, IHC can also be used to directly demonstrate PrP^{Sc} in scrapie-infected tissues [43-45]. PrP^{Sc} immunoreactivity has been found to increase along with increasing GFAP levels, though not necessarily with equivalent distributions [42]. However, severely affected retinas stain intensely and diffusely for both PrP^{Sc} and GFAP [42]. In minimally affected samples, retinal PrP^{Sc} appeared first in the inner and outer plexiform layers. PrP^{Sc} immunoreactivity then extended into the ganglion cell layer, optic fiber layer, and was also expressed in both the inner and outer nuclear layers. One study noted that the highest PrP^{Sc} immunoreactivity levels were seen in the retinal ganglion cell layers, and also correlated retinal lesions to concurrent histopathologic disease in cephalic tissues [45]. Thus animals confirmed to be infected with scrapie upon detection of characteristic lesions in the brain also exhibited retinal pathology.

Fluorescence spectroscopy has also been correlated with PrP^{Sc} accumulation in the retina of scrapie-infected sheep [46]. This work found increases in fluorescence from scrapie-inoculated sheep versus non-infected sheep using fluorescence spectroscopy [46].

While scrapie is not seen commonly in goats they are known to be susceptible. However, because it is not often seen, literature discussing scrapie in goats is limited, and discussion of associated retinal pathology is sparse. IHC showing PrP^{Sc} accumulation in the

absence of other retinal pathology has been reported in goats infected with scrapie, though this was thought to be preceding retinal degeneration [47].

Scrapie in rodents

As previously discussed, prion diseases do not often cross species barriers, and so the development of strains of scrapie that were infectious to rodents has proven invaluable in TSE research. Mouse-adapted scrapie strains in have allowed mapping the disease process in cephalic tissues [19-21], and similarly many studies have shown changes in the retina that have been attributed to PrP^{Sc} protein accumulation.

Early studies of scrapie in rodents often involved hamsters, using a strain of scrapie called 263K. Histopathologic results showed similar findings to those seen in sheep as retinal degeneration was notable, progressive, and most severe in outer retinal layers [37-38,48-51]. Electron microscopy also has been used to evaluate hamster retinal tissues following infection with scrapie. This again showed that progressive retinal degeneration occurred, was most severe in outer retinal layers, but occurred after prion protein accumulation [50]. This supported suspicions (though did not necessarily prove) that prions could be directly retinotoxic, and that retinal degeneration was not necessarily subsequent to generalized neurodegeneration.

Subsequent studies explored the retinal pathology in both hamster and mouse eyes inoculated with 263K scrapie. Interestingly, hamster eyes were noted to accumulate significantly more prion protein than was observed in mouse eye tissue [28,50].

However, one method to circumvent these differential findings was to use transgenic mice expressing hamster PrP. In studies which used this method, tissues were analyzed after terminal stages were reached for each of the following groups: one expressing hamster prion proteins, one expressing hamster GFAP, and one expressing hamster neuron-specific enolase (NSE) [29-30]. After intra-ocular inoculation with a hamster-adapted 263K scrapie the only group to develop retinal pathology was that which expressed hamster prion protein, further supporting the theory that retinal degeneration in TSEs was directly related to PrP^{Sc} replication and accumulation within the eye. In subsequent work the activation of astroglial cells in scrapie-inoculated retinas was also noted [29].

Mouse-adapted forms of scrapie were eventually developed and histopathologic lesions were observed to follow a similar pattern to results previously reported. A study using 79A scrapie (passed only through mice) found that the most severe retinal degenerative changes occurred in the outer nuclear layers, as had been previously observed with scrapie in sheep [52]. Conversely, when ME7 scrapie was inoculated directly into the eyes of mice, the most significant damage occurred in the innermost retinal layer (the retinal ganglion cell layer) while leaving amacrine cells and interneurons largely unaffected. This was interpreted to suggest PrP^{Sc} spread unidirectionally (either retrograde or anterograde) dependent on route of inoculation [31].

Scrapie and other TSEs have also been shown to produce retinal lesions in a number of natural and experimental hosts. In addition to scrapie, other examples include CWD, BSE, vCJD, transmissible mink encephalopathy (TME), and feline spongiform encephalopathy (FSE) [53-58].

Time-course studies

While pathology has been well documented in TSEs following completion of the incubation period (e.g. as seen with documentation of lesion profiling described previously), it is also important to note how disease progresses over time. During the incubation period, while disease is often clinically silent, lesions are often detectable on post mortem examination.

General TSE pathogenesis often begins with ingestion of the infectious particles, these particles then pass transmucosally via mechanisms that are not entirely described, but may involve M-cells of follicle-associated epithelium [59]. Prions then replicate in lymphoid tissue draining the gastrointestinal tract, and eventually enter the nervous system through enteric parasympathetic and/or sympathetic innervation [60]. From there prions can travel to the CNS, first reaching the spinal cord and eventually the brainstem, cerebellum, and cerebrum [60]. Only after the infectious PrP^{Sc} particles reach the brain do they spread via retrograde transport to the retina via the optic nerve. Though this pathogenesis is largely accepted, events can be difficult to document over time, especially in early stages. However, a number of studies have attempted to characterize TSE pathogenesis over time.

Many time course studies have been devoted to detection of the infectious agent in tissues, rather than documenting the pathology of disease over time [61-63]. For example, quantitative assays such as serial protein misfolding assays (sPMCA), and real-time quaking-induced conversion (RT-QuIC) studies have involved serial time points in efforts to measure the respective infectious agents in tissues. Prion protein that is increased in beta sheet content over time in the dorsal root ganglia of infected animals has also been used to document spread from the enteric nervous system into the CNS [64]. The results from these studies demonstrated that

accumulation of infectious prions increased over time, and also confirmed that intracerebral inoculations proved more severe (i.e. accumulated more infectious prion agent) than other routes [61-62].

Other studies have focused on documenting pathology and/or PrP^{Sc} in infected tissues. In sequential-kill studies using 2-month collection intervals, PrP^{Sc} immunoreactivity has been documented in the brainstem prior to development of spongiform lesions at the obex in BSE infected cattle (though only after a dormant period lasting >1 year) [65]. Other work involving the entire cerebrum, cerebellum, and brainstem has shown little to no change in PrP^{Sc} accumulation and GFAP immunoreactivity in early time points, but drastic increases in mid-late stages of disease in murine models of scrapie and BSE [18].

Still other studies have noted disease progression over time in nervous, lymphatic, and other tissues in lesser-known TSEs, including TME [66].

However, almost no evidence exists in the literature for studies that have evaluated changes in the retina over time, especially with documentation of prion accumulation in conjunction with retinal pathology.

Conclusions

Prions are novel pathogens that are unique in their ability to replicate without use of nucleic acids. The diseases they cause are invariably fatal, and some forms of TSEs are transmissible to humans, making research into effective antemortem diagnostics critical. Retinal pathology has been documented in almost all TSEs, and may provide a means for antemortem diagnostics and/or animal screening tests at a herd level to guard against potential

zoonotic transmission. Though prion diseases have been extensively researched, there is a lack of documentation of the course of disease as it occurs in the retina from the time of inoculation to terminal stages. Furthermore, it will also be useful to establish if and how pathology in the retinas varies with a direct inoculation into the CNS (intracerebral) versus a more peripheral route (e.g. intraperitoneal), as multiple inoculation routes have been used extensively studied in TSE studies.

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CHAPTER 3: TIME-COURSE STUDY OF RETINAL PATHOLOGY IN C57BL/6 MICE INFECTED
WITH RML SCRAPIE

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Abstract:

Transmissible spongiform encephalopathies (TSEs) are caused by proteinaceous pathogens known as prions. These diseases develop slowly as the misfolded and protease-resistant prion protein, PrP^{Sc}, interacts with the normal cellular form, PrP^C, a cell-surface protein found throughout the nervous system. This interaction causes the normal isoform to refold into PrP^{Sc} and accumulate within neurons, causing widespread degeneration. PrP^{Sc} accumulates in the retinas of many animals with TSEs. This accumulation and associated pathology may prove to be valuable for non-invasive, pre-mortem diagnostics. Our study aimed to investigate the course of retinal TSE pathology, using a mouse-adapted strain of the scrapie agent (RML) in C57Bl/6 mice. Mice were inoculated with RML-infected brain homogenate,

either intracerebrally or intraperitoneally. Animals were euthanized and tissues collected every 30 days throughout the disease course, then prepared for standard histopathologic and immunohistochemical analysis. Immunohistochemistry was done on retinal tissues using antibodies against PrP (monoclonal antibody 6C2) and glial fibrillary acidic protein (GFAP), which is upregulated under conditions of retinal stress. Cell counts of the outer nuclear layer were done to quantify retinal degeneration. We found retinal PrP^{Sc} accumulation, stress, and degeneration are detectable after approximately 80% of the incubation period in mice, for all detection methods and inoculation routes. However, the associated pathology was somewhat lessened and the incubation period was prolonged in intraperitoneally-inoculated animals. This model will allow for more detailed study of PrP^{Sc} accumulation and neuronal cell death.

Keywords: glial fibrillary acidic protein, immunohistochemistry, prion, TSE, retina, scrapie, transmissible spongiform encephalopathy

Introduction:

Transmissible spongiform encephalopathies, or TSEs, are invariably fatal neurologic diseases caused by interaction of an infectious prion protein, PrP^{Sc}, with its endogenous, normal cellular isoform, PrP^C, a cell-surface protein found throughout the nervous system[1]. This interaction causes a conformation change within PrP^C, as a portion of an alpha-helix is refolded into a beta-pleated sheet. This alters the biochemical and physiological properties of the protein into the infectious PrP^{Sc}, which then accumulates within neurons causing widespread neuronal degeneration, evidenced by characteristic spongiform lesions [2,3]. This replication is unique among pathogens in that it requires no nucleic acid, and can take years to progress to

manifestation of clinical signs. Prion diseases have been described in a number of species, including humans. Scrapie, the oldest known TSE, affects sheep and goats; others include chronic wasting disease (CWD) of cervids, Creutzfeldt-Jakob disease in humans, transmissible mink encephalopathy (TME), and bovine spongiform encephalopathy (BSE)[4,5,6].

TSEs have much in common with other protein-accumulating neuropathies, e.g. Parkinson's disease, Alzheimer's disease, and Huntington's disease [7]. However TSEs are distinct in having infectious forms, which allow for direct inoculation from infected individuals to naïve animals. This distinction among prion diseases can be exploited for research purposes and enables study of the common underlying pathogenic mechanisms from the earliest stages of disease. In particular, scrapie incubation time and pathologic profiles are well-defined in mouse models [8, 9].

The purpose of this study was to compare retinal pathology associated with two experimental routes of scrapie inoculation: intracerebral inoculation (IC) to intraperitoneal (IP), and for these purposes we used the RML scrapie agent injected into C57Bl/6 mice. Retinal degeneration and/or accumulation of misfolded PrP^{Sc} has been documented in a number of TSEs including: scrapie in sheep [10-14], chronic wasting disease in elk [15], transmissible mink encephalopathy [16], feline spongiform encephalopathy in cats [17], in addition to BSE in cattle [18] and variant Creutzfeldt-Jakob's disease in humans [19]. Due to this commonality of prion-associated retinal pathology, coupled with the fact that it is possible to monitor eye function in live animals using modalities such as optical coherence tomography [20,21], the hope is that this study will allow for development of retinal scans as a non-invasive, diagnostic tool for TSEs.

Here we have shown retinal PrP^{Sc} accumulation, stress, and degeneration are detectable after approximately 80% of the incubation period in mice, for all detection methods and inoculation routes. However, disease severity was slightly lessened and progression was prolonged for intraperitoneally-inoculated animals.

Materials and methods:

Ethics Statement: This experiment was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, Champaign, IL). The protocol was approved by the Institutional Animal Care and Use Committee at the National Animal Disease Center (protocol number: 3985) and Iowa State University (protocol number: 7154).

Fifty-one C57 Bl/6 mice aged 6-8 weeks were inoculated with brain homogenate containing mouse-adapted RML scrapie either via intracerebral (IC) injection into the right cerebral hemisphere at the level of the temporal bone, or intraperitoneally (IP) into the ventral abdomen. For both the IC and IP inoculation groups, subgroups were euthanized by use of carbon dioxide chambers at set time points for tissue collection. For IC-inoculated mice euthanasia and tissue collection occurred at approximately 30, 60, 90, 120 days post-inoculation (dpi), along with another group comprising animals exhibiting clinical signs around 155 dpi. For IP-inoculated mice the time points were 30, 60, 90, 120, 150 dpi, and animals that were allowed to progress to symptomatic disease until approximately 200 days post-

inoculation. Furthermore, for each age group and inoculation type there were 2 groups of age matched control mice, one injected with brain homogenate from healthy mice, and the other received no injections.

Upon collection globes were collected into Bouin's fixative. Tissues were then embedded in paraffin for processing, sectioned at 4 microns, and affixed to slides for H&E staining and IHC.

Single label immunohistochemistry was performed using two primary antibodies: GFAP (Dako) and 6C2 (Central Veterinary Institute-WUR), which are described in further detail in the following paragraphs. Both primary antibodies were diluted in blocking solution consisting of 0.1% bovine serum albumin and 1.5% normal goat serum dissolved in 1X tris-buffered saline+ 0.05% polysorbate 20. To determine ideal antibody concentration for immunoreactivity and visibility of labeling, dilution curves were performed for optimization. Primary antibody detection was performed with Dako's HRP polymer (goat anti-mouse/rabbit utilizing horseradish peroxidase). The chromagen 3,3'-diaminobenzidine (DAB) was used for viewing, and hematoxylin was used as a counterstain. 1X tris-buffered saline solutions with 0.05% polysorbate 20 were also used for intermediate washes.

Retinal degeneration was measured by counts of cell bodies spanning the outer nuclear layer (ONL) of the retinas, made visible by hematoxylin stain. For each cross section, three counts were made in the central retina and averaged for a single value for each animal

To assess the retinal response to stress, a polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) was used at a 1:100 dilution in the retina. GFAP is a cytoskeletal protein upregulated by retinal Müller glia under stressed conditions [22]. Endogenous peroxidase in

tissues was blocked by holding slides in a 3% hydrogen peroxide solution with a tris-buffered saline diluent for 20 minutes. Primary incubation time was one hour, secondary incubation for primary detection was 20 minutes, and DAB was left on for 5 minutes.

To detect the levels of PrP^{Sc} in the eyes, mouse monoclonal anti-PrP 6C2 was used as the primary antibody, at a dilution of 1:2000. Because the antibody would not be able to distinguish between PrP^C and PrP^{Sc}, the samples were autoclaved while suspended in distilled water at 121°C for 20 minutes to inactivate the normal cellular form without affecting the heat-resistant infectious prion. Endogenous peroxidase was blocked by leaving tissues in a solution of 3% hydrogen peroxide in methanol for 10 minutes. Primary incubation lasted 2 hours, while secondary incubation lasted 30 minutes, and DAB was applied to eye tissues for 2 minutes. A scale from -1-3 was devised to account for some level of background present in controls. Any tissue exhibiting lesser or equal amounts of immunoreactivity to the negative control showing the most background labeling was assigned a score of 0 or lower. A score greater than zero indicated a significant level of PrP^{Sc} labeling, and when this extended through all layers of retina the sample was assigned a maximum score of 3.

Results were analyzed for significance using one-way ANOVA (analysis of variance) ($p < 0.05$, confidence interval of 95%).

Results:

Retinal degeneration

Thinning of the outer nuclear layer was noted in mice euthanized after showing neurologic disease. To quantify this retinal degeneration over the time-course of the study,

outer nuclear layer (ONL) thickness was assessed by the number of cell bodies present. Non-diseased retinas obtained from positive and negative control mice exhibited ONL thicknesses of 10 cell bodies or greater ($\mu= 11.09$).

Degeneration occurred at earlier time points in intracerebrally-inoculated animals, as compared to intraperitoneally-inoculated animals, but still did not exhibit significant decreases in ONL width until 30 days prior to the terminal time point (120 dpi). IC mean ONL width remained equivalent to control levels until 120 dpi. After 120 days ONL widths dropped to a mean of 8.94 cell bodies, and then fell to a mean width of 2.80 cell bodies at end time points (Figure 1).

The IP group did not show a significant decrease until development of clinical signs at the final time point. IP-inoculated mice displayed a more prolonged incubation time, and degeneration was not seen until 150 days or later with a mean ONL width of 6.08 cell bodies at clinical stages (Figure 2).

PrP^{Sc} accumulation in the retina

To investigate the time-course of PrP^{Sc} accumulation in the retina, immunohistochemistry was used to label misfolded prion protein in fixed tissues. PrP^{Sc} immunoreactivity was minimal for both inoculation routes until at or near clinical stages of disease, at which time retinal tissues were often diffusely labeled. Punctate labeling was occasionally visible as early as 90 days post inoculation in intracerebrally inoculated mice in the photoreceptor layer and outer nuclear layer (Figure 3). However this was not a consistent finding and was seen even more rarely in intraperitoneally inoculated mice at >120 dpi.

PrP^{Sc} immunoreactivity was quantified using a subjective scoring system from -1 to 3. Zero was the score of the tissues with maximum non-specific background staining. Three was the maximum specific PrP^{Sc} immunoreactivity. IC-inoculated mice showed significant accumulation of PrP^{Sc} at 120 dpi, 30 days prior to showing clinical signs. The mean anti-PrP immunoreactivity score at the 120 dpi time point was 0.94 and at clinical levels was 1.47 (Figure 4). Half of the mice in this subgroup exhibited maximal PrP^{Sc} labeling, with scores of 2.0.

In IP-inoculated mice significant and diffuse PrP^{Sc} accumulations did not occur until clinical stages (approximately 200 dpi) (Figure 5). At the clinical stage, 200 days after inoculation, levels of infectious prion accumulation in the retina approached levels seen in IC-inoculated clinical mice, with a mean score of 1.38. Half of the IP-inoculated mice exhibited maximal PrP^{Sc} scoring as well. These results were well contrasted by control scores ($\mu = -0.75$).

Retinal response to stress

Anti-GFAP labels an intermediate filaments present in astrocytes and Müller glia endfeet in a healthy retina, and is upregulated in activated the Müller glia in the diseased retina. In this regard they serve as an effective indication of the retinal response to PrP^{Sc} accumulation and can be seen spanning all layers of the retina when activated. Throughout all time points occasional GFAP-positive Müller glia were visible at the peripheral retina and astrocytes were confined to the optic fiber layer with little variation until clinical stages, when numerous GFAP-positive Müller glia could be seen spanning all layers in the central retina (Figure 6). Incomplete labeling (faint and/or not fully spanning the retina) of Müller glia in central retinas could be

detected in some animals at time points just prior to the clinical stage for both IP- and IC-inoculated animals, but was not a consistent finding.

Discussion:

Our objective for the current study was to document the course of a TSE in a mouse model and compare direct intracerebral inoculation to intraperitoneal inoculation. This was achieved, not only showing that retinal degeneration occurs in TSE-infected mice, but that this is closely associated with increasing accumulation of PrP^{Sc} in the retina. The development of pathology was delayed in intraperitoneally inoculated mice as compared to those receiving intracerebral inoculations. However, the levels of accumulation of PrP^{Sc} in IP-inoculated mice occurring at clinical stages approached levels seen in IC-inoculated mice, and for both inoculation routes disease progression was much more rapid at later stages, 120 days-post inoculation or longer.

The most significant changes in PrP^{Sc} accumulation, retinal degeneration, and evidence of a stress response occurred in the last 30 days of the time-course. This proved true for both IC- and IP-inoculated animals, and IP-inoculated mice did not display substantial pathology until clinical stages. For either inoculation route, at end stages retinal deterioration was severe and PrP^{Sc} accumulation was extensive. Activated Müller glia were absent in the central retina until at or near clinical stage disease, as demonstrated by IHC with anti-GFAP.

Previous research using scrapie passed through mice has shown intraperitoneally inoculated mice develop retinal lesions at later time points than intracerebrally inoculated mice [23]. Intracerebral inoculations in fact have consistently produced more severe disease and

shown a higher degree of prion accumulation in the retina and other tissues, when compared to other routes in TSE studies done with various species [24, 25].

The accumulation of PrP^{Sc} in the retina was only somewhat consistent with previous results. In prior studies of scrapie-associated retinal pathology, PrP^{Sc} labelling was first detected in the inner and outer plexiform layers and then extended into the outer nuclear layer, inner nuclear layer, photoreceptor layer, and ganglion cell layer with the highest accumulation seen in the ganglion cell layer [26,27]. Our work showed very little accumulation in tissues prior to diffuse labeling, but when present punctate PrP^{Sc} labelling first appeared in the PRL or ONL, and the most intense labeling occurred in the ONL.

However, retinal response to stress, as measured by GFAP expression by astrocytes and Müller glia was consistent with previous results. GFAP has been previously demonstrated to increase in immunoreactivity in the retinal Müller glia cells in scrapie inoculated animals, with severely affected retinas showing diffuse labeling [26-28]. Our results showed increased GFAP labeling and activation of Müller glia to occur concurrently with PrP^{Sc} accumulation and retinal degeneration; all of which increased rapidly at or near terminal stages.

This time-course study work has documented how pathology develops in the retina over time, using a mouse model. While most changes were not significant until approaching the end of the incubation period, this narrows the window for future studies of retinal effects in more detail. We have also demonstrated differences in intraperitoneally inoculated mice and intracerebrally inoculated mice and how the two routes compare at different time points. In conclusion this work may provide groundwork for later studies in natural hosts.

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Figures:

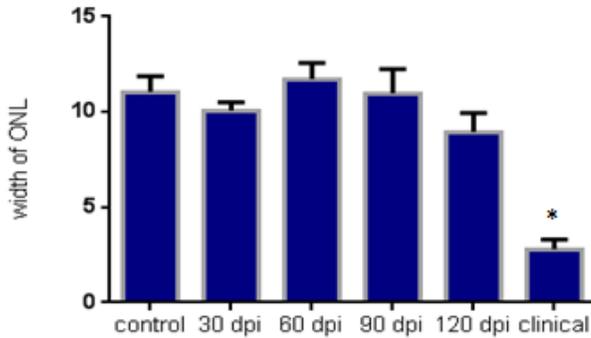
Retinal degeneration in mice inoculated intracerebrally shown by decreasing ONL width

Figure 1- Retinal degeneration in intracerebrally inoculated mice was demonstrated by decreasing width of outer nuclear layer, measured by counts of cell bodies. Significant decreases were not seen until 120 days post-inoculation and clinical stages. * $p < 0.05$, one-way ANOVA

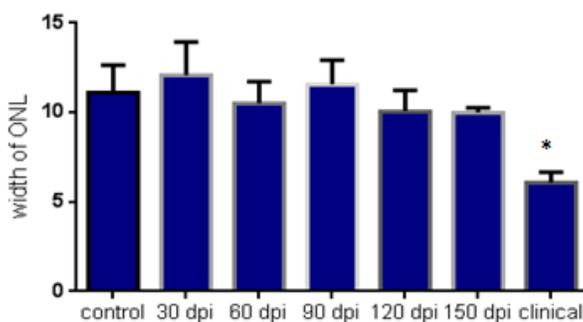
Retinal degeneration in mice inoculated intraperitoneally shown by decreasing ONL width

Figure 2- Retinal degeneration in intraperitoneally inoculated mice was demonstrated by decreasing width of outer nuclear layer, measured by counts of cell bodies. Significant decreases were not seen until clinical stages. * $p < 0.05$, one-way ANOVA

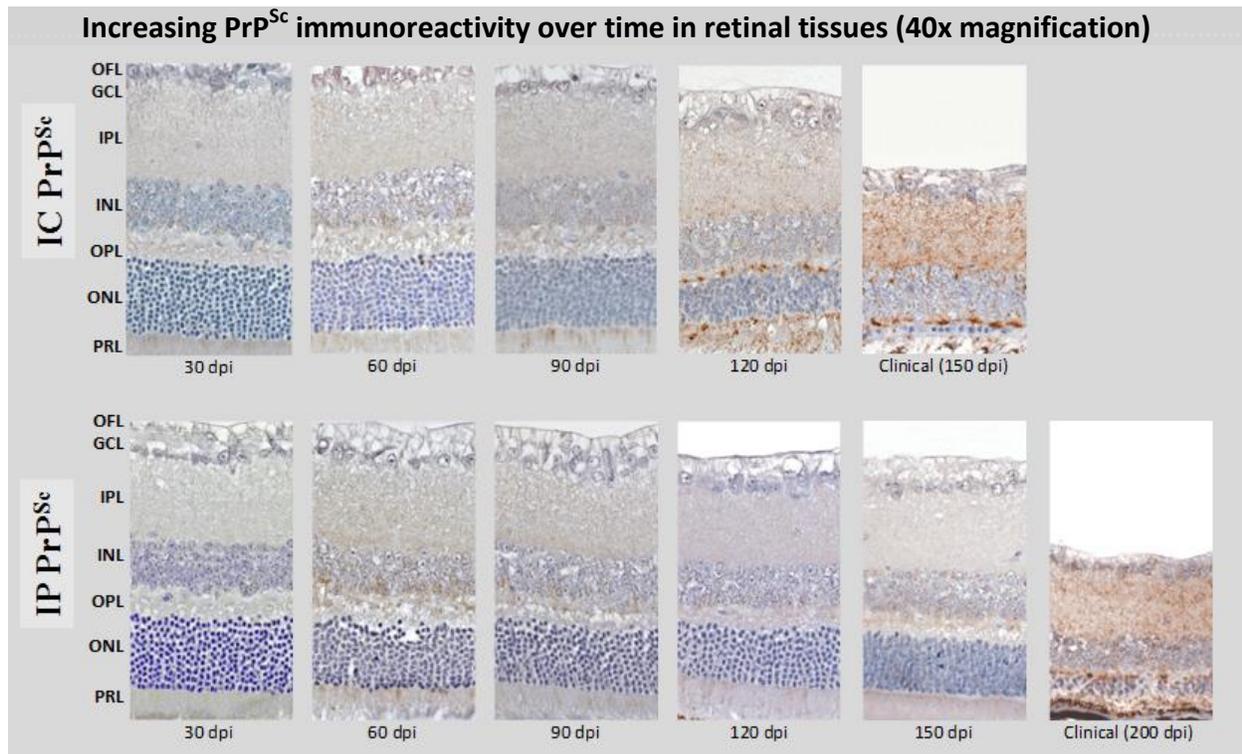


Figure 3- Increasing accumulation of PrP^{Sc} displayed by intensity of immunoreactivity on intracerebrally (IC) vs. intraperitoneally (IP) inoculated animal tissues. Again, IC-inoculated mice developed pathology more quickly than IP. Accumulation of PrP^{Sc} was greatest between the outer nuclear layer (ONL) and outer plexiform layer (OPL).

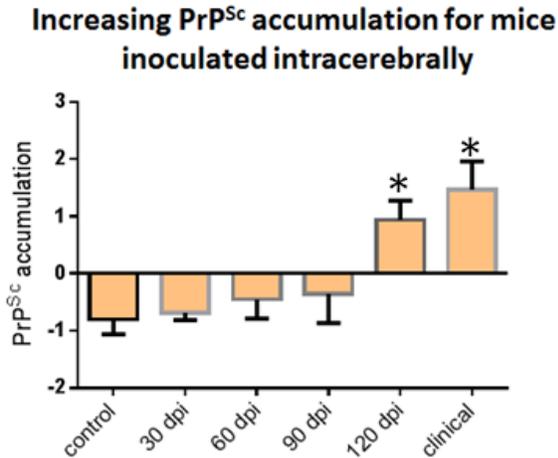


Figure 4- Increased retinal accumulation of infectious prion protein was displayed by intensity of immunoreactivity of IHC on IC-inoculated tissues. Scores were assigned based on intensity of labeling, and significant accumulation PrP^{Sc} was shown at 120 days post-inoculation and clinical stages for intracerebrally inoculated mice. * $p < 0.05$, one-way ANOVA

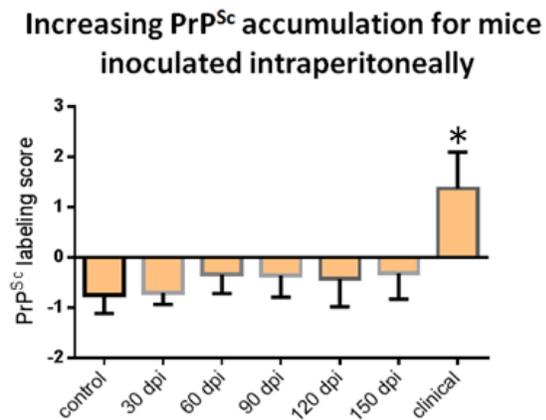


Figure 5- Increased retinal accumulation of infectious prion protein displayed by intensity of immunoreactivity. Scores were assigned based on intensity of labeling, and significant accumulation PrP^{Sc} was shown only clinical stages for intraperitoneally inoculated mice.

* $p < 0.05$, one-way ANOVA

Demonstration of increase in GFAP expression by Müller glia over time and progressive retinal degeneration in tissues (40x magnification)

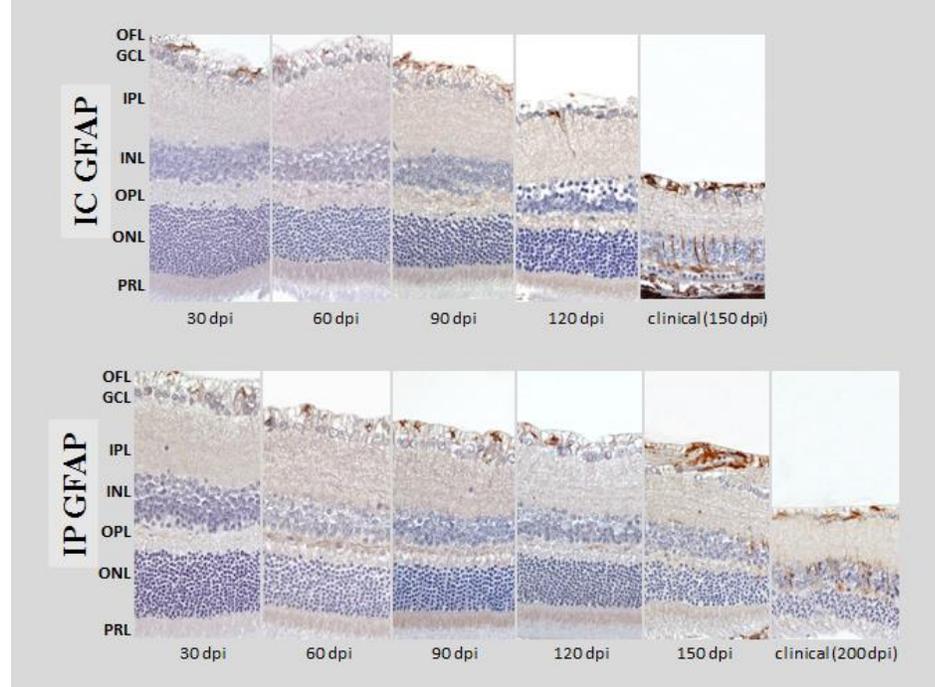


Figure 6- Progression of retinal degeneration and GFAP-positive Müller glia for intracerebrally (IC) and intraperitoneally (IP) inoculated mice. Disease progressed more quickly with an IC route of inoculation than with an IP inoculation route, but neither showed significant changes until near clinical stages.

CHAPTER 4: GENERAL CONCLUSIONS

Summary

Prion diseases have been around for centuries, and yet the etiologic agent was only recently discovered. These are unique, neurodegenerative syndromes that are invariably progressive and fatal. The possibility for transmission to humans exists, making research into development of effective screening tests for animals entering the food chain all the more important. Attempts at pre-mortem TSE diagnostics have thus far proven difficult, especially tests that can be used efficiently and economically on a large scale. Thus the retina has potential for development of such premortem diagnostics, as it is an extension of the CNS which is readily viewable without requiring invasive or even sacrificial sampling. It is possible that documentation of histopathology in the retina over the incubation of disease could provide useful information for development of retinal scans which could screen for disease.

TSEs have been studied in various species, and a great deal of experimental work has relied on hamster or mouse models using many different inoculation routes. It has been demonstrated that scrapie (and other TSEs) can be reliably passed through mice, and shows resultant PrP^{Sc} accumulation and vacuolar lesions in CNS tissues. Additionally, with many TSEs retinal prion accumulation and subsequent (or concurrent) retinal degeneration is known to occur.

Though prion diseases have been substantially researched over the past 2-3 decades, there is still much to learn. While many TSE studies have documented retinal lesions, and even accumulation of PrP^{Sc} establishing retinal pathology alone has not provided a complete picture.

As has been alluded to in previous sections, little information exists as to when exactly pathology becomes detectable in the retina. Our study aimed to investigate this in further detail.

Our results showed retinal pathology did not occur until 80% of the incubation period had passed, and also that disease progressed more slowly in intraperitoneally (IP) inoculated mice as compared to those receiving intracerebral (IC) inoculations. The levels of accumulation of PrP^{Sc} in IP mice occurring at clinical stages did still approach levels seen in IC mice, indicating that progression of disease was initially slow and more rapid at the later stages, regardless of inoculation route. This is consistent with prior research comparing IC inoculation to other routes.

Unfortunately, 30 day time periods were not adequate to determine the exact spread of prion accumulation in the retinas of scrapie-affected mice. Though some pinpoint lesions appeared in the photoreceptor layer and outer nuclear layer, it was difficult to say with certainty which areas the prions localized to first. Still, in later stages the most intense PrP^{Sc} immunoreactivity often occurred at the edge of the outer nuclear layer and/or within the photoreceptor layer, which differed somewhat from previous studies of PrP^{Sc} in scrapie inoculated sheep.

Marked accumulation of PrP^{Sc} did not reach substantial levels until, at most, 30 days prior to development of clinical signs. This is also the pattern seen with retinal degeneration as demonstrated by the decreasing width of the ONL. The width of the ONL was only significantly decreased at clinical stages. Additionally activated Müller glia were not detected in the central retina until at or near clinical stages of disease.

Recommendations for further research

Further studies may have more definitive results with decreased intervals between time points. Furthermore, given documentation of lowered PrP^{Sc} accumulation in mouse retinas compared to hamsters, this may also have impaired study of PrP^{Sc} accumulation in rodent eyes.

In any case, prion research remains relevant today, even though BSE and vCJD cases have drastically declined. Information obtained from TSE studies could also prove valuable to study of other, non-infectious protein misfolding neuropathies. Though not infectious, this list also includes a number of progressive, neurodegenerative diseases for which there is no cure, and diagnosis can often times be inexact. These include Alzheimer's disease, Parkinson's disease, dementia, and others which affect millions of people worldwide.

Concluding remarks

We have described PrP^{Sc} accumulation and associated retinal degeneration in a murine TSE, providing a model in which to study the relationship between PrP^{Sc} accumulation and neuronal cell death in more detail. This allows us to move forward with similar investigations in other species to improve knowledge of TSE pathology and improve diagnostics for this class of diseases, and other protein-misfolding diseases.