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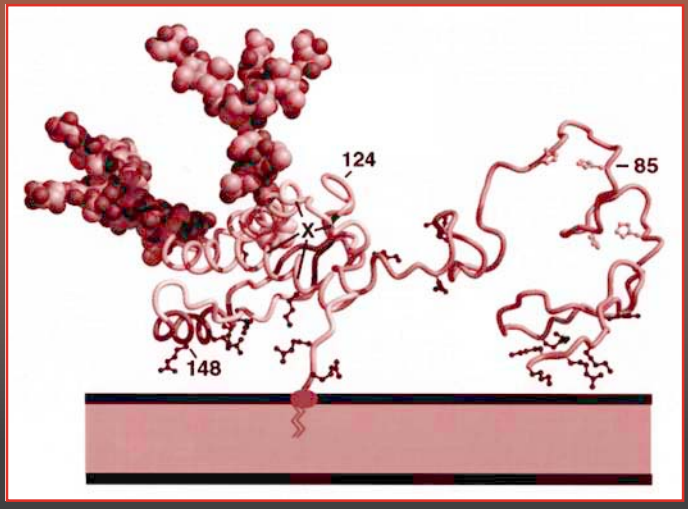
Molecular Pathology of the Prions

Edited by

Harry F. Baker



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Preface

In an earlier volume of the Methods in Molecular Medicine series entitled *Prion Diseases* (1996), Ros Ridley and I assembled contributions from distinguished scientists in order to deliver a comprehensive protocols book that would include every aspect of prion disease research. This well-reviewed book covered human and animal prion diseases with particular emphasis on the methods used in epidemiological study of these diseases and the laboratory-based techniques for analyzing infectious material. Other volumes in the *Methods in Molecular Medicine* series described experimental protocols in such detail that competent scientists could use them to carry out similar experiments. However, because of the wide-ranging subject matter in *Prion Diseases*, and perhaps because of the hazardous nature of the experimental work, we decided to "break the rules" and not to commission a recipe book. Rather, we asked the contributors to describe their different approaches to the various problems that beset our understanding of prion diseases and, though some authors described their experimental techniques in detail, others provided a more general overview of their research.

In the *Molecular Pathology of the Prions*, I have deviated even further from the initial concept of a protocol book. I do, however, think that it follows on from the previous volume and, although the principal authors are different, many of those who contributed to *Prion Diseases* will be found among the bylines here. There is a major difference between the two books. In *Molecular Pathology of the Prions*, I have concentrated on the molecular pathogenesis of prion disease and the emphasis is on the role of prion protein. There is no mention of the epidemiology of animal and human prion diseases that figured so prominently in the earlier book. As a result of the veterinary epidemiological studies carried out by Wilesmith and his colleagues (and so well described in *Prion Diseases*), the measures put in place to curb the epidemic of BSE seem to have worked and the BSE epidemic in Great Britain is almost over. Now, concerns about BSE have been replaced by a growing fear that there will be large numbers of cases of new variant CJD as a result of the consumption of BSE-infected meat. More than 80 people have died from this form of CJD and, despite the widely varying predictions by mathematical modelers, we have no idea how many more will succumb.

There has been a major shift in the status of the prion hypothesis since that earlier volume. This hypothesis, which in its simplest manifestation says that the transmissible agent in the prion diseases is composed solely of prion protein, which has gained more widespread acceptance than it had in 1996, and its originator, Stan Prusiner (who provided the Foreword to *Prion Diseases*) was awarded the 1997 Nobel Prize for Physiology or Medicine. However, although most researchers in the field now subscribe to this hypothesis, it has become clear that in its simplest form it cannot account for all that is known about the prion diseases, and there are those who still espouse the view that the transmissible agent must comprise more than prion protein. All agree, however, that prion protein plays a key role in the molecular pathogenesis of these diseases, and the contributors to the present volume are in the front rank of those investigating this role.

In the first chapter, my colleague Ros Ridley gives an account of the way in which hypotheses develop in general and scientific consensus is constructed. She describes some of the received wisdom about prion diseases (some correct, some incorrect) and how this has influenced the development of the framework within which experimental investigation of prion disease is carried out.

Hans Kretzschmar et al. (Chapter 2) and David Brown and Ian Jones (Chapter 3) review their experiments to elucidate the normal function of prion protein and, in particular, its role as a copper-binding molecule, which acts to regulate synaptic function (Kretzschmar et al.) or cellular resistance to oxidative stress (Brown and Jones).

In Chapter 4, David Brown addresses the neuronal death that occurs in prion diseases. He offers a possible explanation derived from studies of the neurotoxicity of a synthetic peptide PrP106-126, based on the prion protein sequence, applied to tissue cultures, an explanation that incorporates a role for microglia and astrocytes.

Of major interest to prion researchers is the issue of strain of agent. One of the mainstays of the argument that the transmissible agent of prion diseases contains a nonprotein component, probably a nucleic acid, is the existence of different strains of agent. These strains are defined in terms of the regional distribution of neuropathology (the lesion profile) and the incubation period from inoculation to illness onset in defined strains of mice. Although the strain properties of an agent usually remain constant on serial passage through different animals of the same mouse strain, occasionally there is a change that results in a new strain appearing, one that has a different lesion profile and incubation time. Some argue that there has been mutation in an informational component of the agent and that the new strain has been selected. Others argue that the different strains can be encrypted by the tertiary structure of the prion protein and that

there is no evidence for an additional nucleic acid component. This discussion is not covered in this volume. However, the properties of different strains of prion disease agent are covered in Chapter 5 by Martin Groschup and colleagues, who look at the characteristics of different scrapie strains by immunochemical analysis of prion protein and in Chapter 6 by Steve DeArmond, who examines the mechanisms by which different prion strains target different brain regions.

In his experiments, Steve DeArmond made use of transgenic mice and a further three chapters are concerned with studies using such mice. In Chapter 7, Glenn Telling describes experiments in which the prion protein gene has been knocked out or replaced and which have provided information about the molecular basis of strain differences and species barriers.

In Chapter 8, Markus Glatzel and colleagues describe the use of prion gene knockout mice in conjunction with intracerebral grafts of normal brain tissue to examine the spread of infection from the periphery to the central nervous system and within the brain.

In Chapter 9, David Harris and colleagues report their studies of cultured mammalian cells (Chinese hamster ovary cells) or transgenic mice carry and express prion-disease associated prion gene mutations.

In the old literature about prion diseases it is often said that there is no immune response; indeed this has become a dogma. But recent evidence suggests that this statement is unwarranted and that there are changes in the immune system, and in Chapter 10, Samar Betmouni and Hugh Perry describe their investigations of the early inflammatory response to scrapie infection in mice and its role in the pathogenesis of disease.

In Chapter 11, Richard Greene introduces a new approach to the study of prion disease, an approach he terms electroneuropathology. Greene has applied some of the established techniques for electrophysiological recording from brain tissue slices to mice with scrapie infection and has found that changes in neuronal activity are sensitive to the combination of agent strain and mouse strain used. He has also reviewed the electrophysiology of hippocampal formation and subiculum in prion protein null mice.

Two chapters are concerned with amyloidosis. It has become clear in the past few years that many neurodegenerative diseases are associated with the accumulation of protein deposits within brain parenchyma. In these diseases a normal cellular or circulating protein is converted to an abnormal β-sheet form, which aggregates to form very stable, insoluble plaques or amyloid deposits. The best-known is β-amyloid, which is found within the plaques that are a prominent feature of the neuropathology of Alzheimer's disease. In Chapter 12, Martin Jeffrey and Jan Fraser review their work on the aggregation and

deposition of abnormal prion protein and its relation to pathological change and disease. Further they briefly discuss tubulovesicular bodies, which might have a role in the pathogenesis of prion diseases. In Chapter 13, Thomas Wisniewski and his colleagues describe their work on "β-sheet breaker peptides", which interfere with the process of amyloidosis, and consider their therapeutic potential in the treatment of neurodegenerative diseases that are associated with amyloid deposition.

In the final chapter (Chapter 14) we move away from mammalian prions to consider prions of yeasts. Reed Wickner and his colleagues, who have been at the forefront of research in this area, give a full account of the nonchromosomal genes [URE3] and [PS1] of yeast that are infectious forms of Ure29 and Sup35p, and that, according to Wickner and colleagues, satisfy the criteria for being considered prions. They argue that studies of yeast prions have cast light on the biology of mammalian prions and speculate that such studies will suggest useful treatments for human prion or amyloid diseases.

This, then, is the subject matter of this volume. I am grateful to the authors who agreed to contribute, despite the heavy demands on their time and efforts in this rapidly growing and increasingly important area of biology.

Harry F. Baker

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1

What Would Thomas Henry Huxley Have Made of Prion Diseases?

Rosalind M. Ridley

1. Introduction

"Science is nothing but trained and organized common sense, differing from the latter only as a veteran may differ from a raw recruit."[a]

Prion disease is a disease of the second half of the twentieth century, but the scientific method that has elucidated this fascinating group of diseases is much older. As an illustration of this, this chapter considers the way in which a nineteenth century scientist might have reacted to the challenge that prion disease has presented. T. H. Huxley (1825–1895) was an ardent naturalist, who traveled around the world collecting specimens, and who peered down the microscope ***(1)***. He amassed vast amounts of data, and could work prodigiously hard. His approach to science can be judged from some of things that he said. He was a confrontational character, and would undoubtedly have joined in the arguments that led to the concept of prion disease, if he had lived a century later.

2. Paradigm Shift and Paradigm Drift

"The great tragedy of science – the slaying of a beautiful hypothesis by an ugly fact."[b]

It has been my privilege to work in an area that has undergone a major 'paradigm shift' ***(2)*** in a period of a few years, a shift exemplified by the change in name, from "transmissible spongiform encephalopathy" (TSE) to "prion disease." This change is critical, because it moves the central, defining feature of

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this type of disease away from clinical features (etiology and neuropathology) toward the recognition of the central role of a particular protein in pathogenesis. This paradigm shift has been exciting, not just because of the impact it has had on understanding these diseases, but also because it casts into sharp relief the process of evolution of ideas and perceptions, which constitutes scientific development. An important feature of this process is scientific consensus, which rests as much on psychological factors, such as perspective, persuasion, and comprehension, as it does on the production of factual data. Kuhn ***(2)*** put forward a view of the process of science as consisting of periods of slow accumulation of experimental results, much of which elaborates current theories, but some of which produces data of such knotty contradiction to the prevailing view that eventually the theoretical edifice falls apart. This opens up the possibility of a move to a new theoretical paradigm, and rapid changes in scientific understanding ensue. All this is true, but in its simplest form it does not take account of the difference between facts and beliefs, the difference between events in the outside world and ideas in the collective mind of the scientific community. What goes on in the latter produces "paradigm drift," in which concepts change their meaning to such an extent that they fail to resemble the ideas of their originators.

Paradigm drift is as important as paradigm shift in the progress of science, but it needs to be recognized for what it is: an essential development of ideas, not a change in the facts of experimental data. Charles Darwin is the most influential of all biologists, and arguably is among the handful of the most important of all scientists. But his influence lies in the very fact that his ideas have undergone radical development in subsequent years. Asked what "Darwinism" means today, many people will mention something about *Survival of The Fittest* as opposed to *The Inheritance of Acquired Characteristics,* a counterview attributed solely to the pre-Darwinian biologist, Jean-Baptiste de Lamarck. Something may also be mentioned about *"Genetics and Mutation,"* ideas more appropriately attributable to Mendel, Fisher, and Haldane, although Darwin did not know of Mendel's work, lived before Fisher and Haldane, attributed the phrase *Survival of The Fittest* to Herbert Spencer [c] and believed that the *"*variability*,"* upon which natural selection worked, arose "from the indirect and direct actions of the conditions of life, and from use and disuse." [d] This clearly means that he thought that changes of form, which had occurred as a consequence of interaction with the environment, were inherited by the offspring of these well-adapted and reproductively successful animals. But so deeply embedded is the notion that Darwinism stands for the opposite of the inheritance of acquired characteristics that the Oxford Dictionary of Quotations excludes the phrase "from the indirect and direct actions of the condi-

tions of life, and from use and disuse" from the paragraph that it chose to quote in order to exemplify what Darwin believed in. In effect, Darwin is being censored for not being sufficiently Darwinian. What Darwin did was to propose a reductionist explanation of evolution, onto which the subsequent study of genetics could be neatly mapped. The fact that Darwinism has undergone so much drift is why Darwin is so important.

Paradigm drift occurs in all science and a brief consideration of the concepts of Creutzfeldt-Jakob disease (CJD) and TSE illustrates the ways in which these terms evolved before they became enveloped in the concept of prion disease. CJD did not appear as an entry in the International Classification of Diseases until 1979, although Creutzfeldt had described his original case in 1920 ***(3,4)*** and Jakob described four cases in 1921 ***(5,6)***. In the intervening period, many authors have described cases that were thought to resemble these early cases, and a disease entity began to emerge by the process of consensus. Many of these cases were collated by Kirschbaum in a monograph entitled *Jakob-Creutzfeldt disease*, published in 1968 ***(7)***. (The process by which Jakob and Creutzfeldt changed places in this appellation is, in itself, an example of the evolution of an idea in the corporate mind). However, the picture had been very confused, and Kirschbaum says that he undertook his review because his colleagues had questioned ''whether the syndrome is more than a convenient dumping ground for otherwise unclassifiable dementias with interesting cross relations to certain systemic degenerations''. Kirschbaum's remarkable book documents many of the possible presentations of prion disease, including cases of rapid onset and progression, ataxic forms, and cases that resemble fatal insomnia. Modern molecular techniques have now rediscovered these forms as part of the spectrum of prion disease, e.g., acute presentation ***(8)***, the ataxic form ***(9)***, and familial and sporadic fatal insomnia ***(10,11)***, although some of these cases fall outside the strict rubric of TSE. Nonetheless, by the neuropathological criteria that have evolved since the 1920s, Creutzfeldt's first case, and two of Jakob's first four cases, did not have CJD ***(12)***. Without wishing to denigrate the contribution of the eponymous authors, it must be acknowledged that many other, less easily identifiable, scientists participated in the emergence of CJD as a disease entity. Thus, Creutzfeldt and Jakob instigated a scientific concensus from which, ultimately, their original cases were largely disqualified.

Another way in which the consensus surrounding TSE has been subject to paradigm drift is the perceived centrality of spongiform encephalopathy to these diseases. Spongiform encephalopathy was not regarded as being a major feature of the original cases of this group of diseases. Hadlow's perspicacious letter to *the Lancet* in 1959 ***(13)***, which pointed out the clinical and neuro-

pathological similarities between scrapie and kuru, mentions ''large single or multilocular soap bubble vacuoles in the cytoplasm'' as a feature of either disease, only as the last of all the similarities. Klatzo et al. ***(14)*** did not comment on spongiform change in 12 early cases of kuru that came to postmortem, and Beck and Daniel's early work found that spongiform change, if present at all, was a minor feature ***(15)***. Subsequently, spongiform encephalopathy came to be regarded as the defining feature of this type of disease, hence the term ''transmissible spongiform encephalopathy''. But, later still, the diagnostic use of prion protein immunostaining ***(16)***, prion protein immunochemistry ***(17)***, and prion gene analysis ***(18)*** indicated that spongiform encephalopathy was not an obligatory feature of cases which were clearly diagnosable as prion disease by other criteria.

The experimental transmissibility of kuru and CJD was demonstrated in the late 1960s ***(19,20)***. This crucial scientific discovery, together with the practical development of the production of mouse-adapted strains of the transmissible agent ***(21)***, opened up the possibility of much experimentation and the production of a great deal of intriguing data, which is still important. But the etiology that transmissibility was thought to imply produced a profound conceptual segregation of these diseases from all the other human neurodegenerative diseases, which delayed the comparison of the clinical variables across the neurodegenerative diseases until a much later time ***(22)***. This, together with the molecular diagnostic techniques referred to above, which identified cases in which transmission was either not attempted or did not succeed, and in which spongiform encephalopathy was not seen, led to the somewhat comical concept of ''nontransmissible, nonspongiform TSE.'' This situation inevitably gave way to the adoption of the term 'prion disease.''

3. Myth and Misunderstanding

''Irrationally held truths may be more harmful than reasoned errors.''[e]

An important aspect of the interpretation of data— the conversion of facts into knowledge—is an understanding of the circumstances in which the data have been collected. Until the development of mouse-adapted strains of agent in the 1960s, work on scrapie was slow, and required field experiments as well as laboratory analysis. Some of these experiments were limited, and were sometimes subsequently subject to a degree of overinterpretation, to the consternation of the original author. For example, Hadlow, who conducted a few experiments on infectivity in the peripheral tissues of sheep ***(23)*** was distressed to find that these data, collected in the context of general interest, were later used as the definitive data on which to base decisions about the possible infectivity of peripheral tissues of bovine spongiform encephalopa-

thy (BSE)-infected cattle, prior to the completion of experiments in cattle ***(24)***. Hadlow said of his own data ''rather than treating them as tentative findings, they are accepted as established facts about the disease; they become part of the scrapie dogma. But sometimes they do not deserve that distinction'' ***(25)***.

One of the most frequently cited pieces of evidence in favor of maternal transmission, as an important factor in the epidemiology of natural scrapie are the reports by Pattison ***(26,27)*** which said that a number of sheep, fed on placental membranes taken from dams with scrapie, subsequently developed scrapie. But Parry (a friend of Pattison) claims that Pattison believed that this route of infection, if it did occur in the field, could account for no more than 5% of field cases of scrapie ***(28)***. In reviewing the role of placental infection in the epidemiology of natural scrapie, Hadlow later remarked of some people, ''For them it is one of the facts about scrapie'' ***(25)***. It remains so, despite the fact that embryo transfer experiments in sheep ***(29,30)***, epidemiological surveys of natural scrapie ***(28,31,32)*** and experimental studies in primates ***(33)*** have failed to detect maternal transmission. Cohort studies ***(34)*** and epidemiological surveys ***(35)*** in cattle have failed to distinguish between common exposure, genetic predisposition, and maternal transmission as an explanation of the excess occurrence of BSE in the offspring of cows that subsequently developed BSE. Hoinville et al. ***(36)*** calculated that, even if this excess BSE were cause by maternal transmission, it would have had a negligible impact on the BSE epidemic in Britain. Kuru was not passed to the children of kuru victims, except by contamination during funerary practices ***(37)*** and the familial occurrence of other forms of prion disease is entirely accounted for by mutations within the prion gene.

Nonetheless, infectivity has been reported in rodent transmission studies from human blood and placenta in various laboratories, which has fueled the view that maternal transmission as a cause of sporadic CJD is both a risk and a fact. But, as Baron et al. ***(38)*** have pointed out, the lack of difference in incubation time between assays using brain, compared to other tissues or bodily fluids, lack of experimental replication, and high levels of unexplained deaths in experimental and control groups, casts doubt on these reports. Failure to transmit from human blood to primates, following intracerebral inoculation (the most sensitive bioassay), would seem to be a more robust finding ***(39)***. Infectivity has been found in blood of experimentally infected rodents (e.g., **refs. *40–42***), but this occurrence in artificial situations may not have epidemiological implications. HIV is carried in blood, and mosquitoes transfer blood from person to person in sufficient quantity to transmit diseases such as malaria, but AIDS is not transmitted by mosquitoes.

''The chess-board is the world; the pieces are the phenomena of the universe; the rules of the game are what we call the laws of nature. The player on

the other side is hidden from us. We know that his play is always fair, just, and patient. But we also know, to our cost, that he never overlooks a mistake, or makes the smallest allowance for ignorance.''[f]

Luck was never going to be on the side of those whose job it was to cope with BSE. It made little difference in terms of planning how to cope with BSE, whether, in the mid-1980s, one accepted the relatively new prion hypothesis or clung to the ''unconventional virus'' view of the TSEs. BSE was a new disease that may not behave entirely like other TSEs. It was clear that doing the experiments necessary to establish the transmissibility, incubation period, species barrier, and tissue distribution of infectivity of this new disease would take at least 5 years.

Meanwhile, attempts had to be made to establish the source of infection (assuming that contamination, rather than inbreeding, was the source of the early cases), and to remove it. But it would not be known for 4–5 yr whether such measures had been successful, and each further attempt to reduce the spread of infectivity between animals would also take a further 4–5 yr to evaluate. In addition to this the agent of prion disease is difficult to destroy, and is infectious at very low doses. Transmission to other animals is still the most sensitive method of detecting infectivity. Despite advances in in vitro tests for prion protein ***(17)***, it is still not possible to demonstrate that tissue, food, or medicinal products contain so little infectivity that no disease will occur when several million cows or people are exposed to it. The number of people infected with new variant CJD cannot be accurately assessed at present ***(43)***. If the first cases of new variant CJD result from exposure early in the BSE epidemic, then the minimum incubation period is about 10 yr ***(44)***. The maximum incubation for kuru amongst the cannibals of Papua New Guinea is in excess of 40 yr ***(45)*** and the same may apply for new variant CJD. Most of the scientists who witnessed the onset of the BSE epidemic in Britain will not know the full extent of the disaster, because they will have died of old age before it can be certain that there will be no more cases of new variant CJD.

4. Development of The Prion Hypothesis

''It is the customary fate of new truths to begin as heresies and to end as superstitions.''[g]

In 1960, Palmer published a paper in which he acknowledged the wholly unusual nature of the scrapie agent, and suggested that it ''may be a non-protein moiety, perhaps a carbohydrate, which on introduction to the body forms a template for the subsequent reduplication of the agent… If the nature of the agent causing scrapie can be finally determined the results may lead to spectacular changes in the present-day concept of the genesis of disease'' ***(46)***.

Palmer was incorrect to dismiss the possibility that the infectious agent could be a protein, but his idea that the agent could act as a template for the formation of more of itself, is central to the current theory of prion replication.

Other authors, notably Pattison and Jones ***(47)***, Griffith ***(48)***, and Lewin ***(49)*** saw that the remarkable resistance of the scrapie agent to physiochemical inactivation ***(50,51)*** implied that it may not contain nucleic acid and proposed that the information-containing and replicating part of the agent may be a protein. Gibbons and Hunter ***(52)*** and Hunter et al. ***(53)*** made the same sort of arguments, but proposed that the agent was a replicating polysaccharide. None of these authors was able to suggest how this replication might take place, although Pattison suggested that, perhaps, ''the scrapie agent is present in an inhibited form in normal tissue and in a released form in scrapie tissue.'' Griffith's contribution ***(48)*** was truly prescient, in that he discussed various possible mechanisms by which infectious disease could arise spontaneously, and information enciphered (to use the modern term [***54***]) in protein structure could be transferred to other protein molecules. First, he suggested that a disease-producing gene may normally be silent, but be expressed during disease. This allowed strain variation, and variation in host susceptibility, to reflect different polymorphisms in the host gene, with concomitant differences in predilection for gene de-repression. Second, he suggested that the protein may take up different conformations, one of which was envisaged as being disease-related, without changes in primary structure. Such a conformational change was an unknown phenomenon at that time. Third he recognized that the ability of proteins to form polymers was another way in which proteins of the same primary structure may have different biological properties.

In 1962, Parry published an article claiming that, despite being experimentally transmissible, natural scrapie was wholly genetic in origin (55). Dickinson et al. (56) replied with the more conventional idea that the pattern of disease was consistent with genetic susceptibility to an environmental agent or maternal transmission of the infectious agent. Parry persisted, and, in 1973 submitted an article to Nature, the last sentence of which read ''the hypothesis most consistent with present evidence is that the scrapie TSEPA [transmissible encephalopathy agent] is formed de novo in each affected animal by the metabolic activity of the natural recessive gene'' (28). His cover letter to the editor said, ''in view of … Dr Gadjusek's Nobel Prize Oration last year, it seems important to place on record facts regarding scrapie in sheep which are generally overlooked in the scramble to establish a primary infectious aetiology for this groups of disorders'' (28). The paper was rejected on the grounds that the conclusions were erroneous. What the word ''erroneous'' meant in this case was not ''incompatible with the evidence,'' but rather ''incompatible with the prevailing view'' a confusion between facts and beliefs. Parry was vilified for

his views by many of the virologists who were working on scrapie (*see* the Foreword by Alpers in **ref.** ***28***) but he had friends among the shepherds with whom he had worked all his life, and who knew that scrapie was associated with excessive in-breeding ***(57).***

These early workers knew that they were dealing with something that was outside the revolutionary developments in molecular genetics in the 1960s. Between them, they had all the essential bits of the jigsaw, but lacked the experimental protocols that were later to allow the ''prion hypothesis'' to be proposed ***(58)***. The prion hypothesis is not heretical to the central dogma of molecular biology—that the information necessary to manufacture proteins is encoded in the nucleotide sequence of nucleic acid—because it does not claim that proteins replicate. Rather, it claims that there is a source of information within protein molecules that contributes to their biological function, and that this information can be passed on to other molecules. But the protein molecules are still manufactured according to the instructions contained in nucleic acid. The additional information source is the conformation or shape of the protein molecule. The conversion of prion protein from the normal cellular form to the disease-associated form involves a conformational change ***(59)***. Furthermore, the abnormal form of prion protein can have one of several different conformations, and these differences explain the existence of the many strains of agent ***(54)*** which for so long were regarded as the main evidence in favor of a nucleic acid based informational system within the infectious agent.

''I am too much of a sceptic to deny the possibility of anything.''[h]

Are prions alive? They contain information enciphered in the shape of the prion protein molecule, and that information is transmissible from molecule to molecule. The information encoded in DNA is transferred in the replication process to the two DNA strands that are manufactured from the unfolding of the one parent DNA molecule. In prion replication, there is no manufacture of new prion molecules, but the principle of information transfer, and therefore information replication, persists. The precise mechanism by which this occurs is still elusive, but it seems to involve the partial unfolding and subsequent refolding of abnormal prion protein molecules, so that contiguous normal prion protein molecules also assume the abnormal conformation. Are there other examples of such self-replicating information? Although computer viruses were invented by computer terrorists, their defining feature is that they contain enough information to direct the computer in which they reside to recreate more of the viral information-containing sequences, and so they behave as self-propagating machine infections. Another example of self-replicating information is that of the spread of ideas within an intra communicating population, i.e., within a culture. The autonomy of ideas as replicable information, whether

they be pieces of factual information or new ways of looking at things, is emphasised in the concept of ''memes'' ***(60)***. By mechanisms that are not entirely understood, the brain modifies its fine structure to store information from outside, and such information can bring about this change in as many brains as it has direct or indirect contact with. From this point of view, prions are not alive like conventional organisms, but they belong to a group of interesting phenomena that comprises not only living organisms, but also other forms of replicating information systems including the propagation of ideas.

Contemporary prions are parasitic on the prion protein manufactured by the host cell, but the mechanism by which the information contained in the shape of the prion protein is imparted to other prion protein molecules does not depend on cellular mechanisms ***(61)***. This mechanism of replication could, therefore, have evolved prior to the evolution of cellular systems, and, because it also does not depend on DNA, could have been at work in the ''primeval soup'' of small polypeptides, which is presumed to have preceded the evolution of life itself. The demonstration that heritable conformational changes can also occur in certain proteins found in yeasts and fungi ***(62,63)*** and possibly widely throughout biological systems suggests that this form of replication may be ancient.

In addition to the evolution of DNA-based replication, another problem of the change from primeval soup to organisms is the change from liquid to solid life forms. It is not enough that the genes contain all the information necessary to make an organism: That organism must also be capable of developmental self-assembly. The process begins with individual molecules that must stick together. The abnormal form of prion protein belongs to that class of proteins capable of forming amyloids ***(64)***. These orderly aggregations of molecules are formed by self-assembly, which often occurs under artificial, as well as natural, conditions. Where these aggregations cause disease, the disease may be regarded as a disorder of molecular self-assembly, a process which is inevitable, given that biology is fallible and self-assembly is obligatory. Abnormal amyloid formation by at least 18 different proteins is associated with disease ***(65)***.

There is simplicity in wishing to confine the term ''prion disease'' to those diseases in which the abnormal form of prion protein can be detected, but, if prions are defined as ''elements that impart and propagate conformational variability'' ***(66)*** then prions have been found in other biological systems, notably yeasts and fungi ***(62,63)***. These discoveries suggest that protein conformational variability may be a widespread component of non-Mendelian inheritance, which would have important biological functions, as well as disease potential. Like mitochondria, which pursue their own genetic destiny within the cell, and ''junk'' DNA, which quietly replicates itself within the genome, an archaic replication mechanism of the primeval soup may be working on its own agenda

within the cytoplasm of the cells of other organisms. This multiplicity of information replication systems is further exemplified at the level of the whole organism. What is quaintly regarded as one organism with one genome carries within itself many obligatory parasites that are essential for the survival of the main organism, and, within the ecological system, all organisms play a role in the survival, as well as the destruction, of other organisms. The biomass is itself composed of many information-replicating systems, but none of them is independent. The concept of "one genome, one organism" is beginning to look less clear cut.

Prions are on the borderline between biology and chemistry. Because of their disease-causing, infectious nature, they have been regarded as a biological problem, and, for many years, they were studied as though they were viruses, or at least unconventional viruses. But their ability to persist outside living organisms, seemingly indefinitely ***(67)***, and their resistance to chemical and physical inactivation by methods that include ashing at 600°C ***(68)***, means that they can also be regarded as environmental pollutants. Prions polluted cattle feed in the 1980s and led to the BSE epidemic in Britain in the 1980s and 1990s. Cattle, cattle-feed, and the ingredients of cattle-feed were exported to Europe and beyond, and BSE is now emerging as a serious problem in Europe. BSE is likely to behave like other new diseases, whether caused by infection or pollution: a high incidence, but geographically confined, effect eventually gives way to a widespread, but low-incidence disease occurrence. Although the early effects of a major disease epidemic may be very dramatic, the widespread and potentially permanent endemic stage of a disease may ultimately claim more lives. The cost of destroying a wide but thinly spread hazard may be much greater than the cost of containing a small but high level of contaminant. BSE is currently confined to countries capable of dealing with it, given the necessary political will. If BSE were to escape to developing countries it would be quite impossible to eradicate it even though the conditions that lead to large outbreaks of disease may not occur in those countries.

6. CONCLUSION

"If a little knowledge is dangerous, where is the man who has so much as to be out of danger?"[i]

The TSEs have produced two Nobel Laureates, Carlton Gadjusek and Stanley Prusiner, both within the decades that saw, in a different arena, the unraveling of the genetic code and its control of cell function. Lewis Thomas referred to scrapie as ''the greatest puzzle in all biology'' ***(69)***, and, from the point of view of the main thrust of molecular biology during that time, TSEs, and subsequently prion disease, were always eccentric. Their very peculiarity

attracted the maverick who could see that these diseases indicated a secret important to understanding all biology. Worrying about the bits of data that do not fit the picture is as important as understanding the way the other bits hang together to produce a coherent whole. Caution as well as audacity is required to get it all right. As Prusiner himself argues, ''In prion research as well as in many other areas of scientific investigation, a single hypothesis has all too often been championed at the expense of a reasoned approach that involves continuing to entertain a series of complex arguments until one or more can be discarded on the basis of experimental data" ***(66)***.

Thomas Huxley was part of the biggest paradigm shift that there has ever been: the battle for the acceptance of evolution as the origin of species. He knew nothing of prion disease, and is unlikely to have heard of scrapie, because the introduction of many cross-breeds of sheep in the nineteenth century had produced a dramatic decline in this disease ***(28)***. He had visited Papua New Guinea as a naturalist-explorer, but had limited access to the island, because the ship's captain was reluctant to land, fearing that the indigenous population were cannibals ***(1)***. The kuru epidemic that decimated some highland tribes in Papua New Guinea less than a century later, and which was almost certainly maintained by cannibalism ***(70)***, suggests that the explorers' fears may have been justified. Huxley was a vehement supporter of Darwin and of the atheistic, bottom-up explanation of our existence that evolution implied. Darwin seemed to win, although a kind of compromise arose in the early part of the twentieth century between scientists and theologians, so that it appeared that evolution and religion were not incompatible. But the elucidation of the precise mechanisms of DNA replication and genetic determinism in the second part of the twentieth century rekindled the row between the bottom-up evolutionary biologists, evolutionary psychologists, and sociobiologists and the top-down theologians, philosophers, and academics of the arts and humanities. The row is still about where the information comes from that drives the structure and behavior of the biological world, including man. In his latest work, *Consilience*, E. O. Wilson is striving to push the domain of the bottom-up explanation of the world beyond individual psychology and into the area of population dynamics: sociology, economics, and ecology ***(71)***. The "selfish genes" ***(60)*** have had great impact in this debate but prion disease has shown that they are not the only replicating information system that can have a bottom up influence. The conformational changes of prion protein have led through cellular dysfunction and fatal disease, to the political, economic, and ecological disasters of BSE, and the personal and social disasters of kuru and new variant CJD. T. H. Huxley would have been in his element in these debates.

Quotes

[a] T. H. Huxley: *Collected Essays*, iv. The Method of Zadig.
[b] T. H. Huxley: *Collected Essays*, viii. Biogenesis and Abiogenesis.
[c] C. Darwin: "The expression often used by Mr Herbert Spencer of the Survival of the Fittest is more accurate, and is sometimes equally convenient." *The Origin of Species*, 1859.
[d] C. Darwin: *Origin of Species*, 1859.
[e] T. H. Huxley: *Science and Culture*, xii. The Coming of Age of the Origin of Species.
[f] T. H. Huxley: *Lay Sermons*, iii. A liberal education.
[g] T. H. Huxley: *Science and Culture*, xii. The Coming of Age of the Origin of Species.
[h] T. H. Huxley: *Letter to Herbert Spencer*, 22 March 1886.
[i] T. H. Huxley: *On Elementary Instruction in Physiology*, 1887.

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Prion Protein as Copper-Binding Protein at the Synapse

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1. Introduction

Various approaches have been taken to study the function of prion proteins. Biochemical methods were applied to search for a binding partner of PrP^C which is attached to the cell surface by a glycosylphosphatidylinositol GPI anchor ***(1)***. The glial fibrillary acidic protein was one of the first possible binding partners to be described ***(2)*** followed by Bcl-2 ***(3,4)***, molecular chaperones ***(5)***, amyloid precursor-like protein 1 ***(6)***, the 37-kDa laminin receptor ***(7)*** and a 66-kDa membrane protein which has not been characterized in more detail ***(8)***. However, it has not been possible to show any biological significance for PrP^C binding of these proteins. Based on biochemical analyses of chicken PrP^C, Harris et al. ***(9)*** hypothesized that PrP^C may play a role in the regulation of the expression of cholinergic receptors at the neuromuscular endplate.

Biochemical, morphological, and electrophysiological studies of the first PrP gene (*Prnp*) knockout mouse ($Prnp^{0/0}$ mouse), which was generated by Büeler et al. ***(10)***, showed a regular expression of the acetylcholine receptor ***(11)***. Except for changes in its circadian rhythm ***(12,13)*** and increased sensitivity to seizures ***(14)***, this $Prnp^{0/0}$ mouse showed no developmental or behavioral changes ***(10)***. These findings were confirmed in studies of another $Prnp^{0/0}$ line generated by Manson et al. ***(15)***. The lack of severe defects in these two lines of $Prnp^{0/0}$ mice was ascribed to adaptation, because PrP^C was absent throughout embryogenesis. However, transgenic mice expressing inducible PrP^C-

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transgenes that were rendered PrP^C-deficient as adults by administration of doxycycline have remained healthy for more than 1.5 yr ***(16)***. A third $Prnp^{0/0}$ mouse generated by Sakaguchi et al. ***(17)*** showed progressive ataxia and loss of Purkinje cells in mice aged more than 70 wk. Also, a fourth independently generated $Prnp^{0/0}$ mouse ***(18,19)*** exhibits ataxia and Purkinje cell degeneration. Weissmann ***(20)*** suggested that additional deletions of intronic sequences of Prnp may play a role in this knockout line. Most recently the upregulation of a novel PrP^C-like protein, designated Doppel, whose gene is located 16 kb downstream of the mouse PrP, has been speculated to be the cause of Purkinje cell degeneration observed in two of the $Prnp^{0/0}$ mouse lines ***(21)***. Even though the hypothesis of the interaction of prion proteins with cholinergic receptors thus could not be confirmed, the studies of Harris et al. ***(9)*** indicated that PrP^C is enriched at the neuromuscular end-plate, i.e. at synaptic endings. Indeed immunohistochemistry of PrP^C-overexpressing transgenic mice reveal a synaptic expression pattern of PrP^C ***(22,23)***. PrP^C is predominantly expressed in regions of high synaptic density, such as the inner and outer plexiform layer of the retina or the cerebellar molecular layer (**Fig. 1**), in contrast to earlier studies in which a predominantly somatic expression of PrP^C was described ***(24–26)***. Further evidence for a preferentially synaptic location of the prion protein in the central nervous system was shown in immunoelectron microscopic studies by Fournier et al. ***(27)*** and Salès et al. ***(28)***. Electron microscopic evidence for a synaptic location of PrP^C has proven very difficult, however. Thus, it was necessary to use embedding techniques leading to destruction of cell membranes. As a consequence, the electron microscopic evidence for PrP^C location in synaptic vesicles has been disputed. Biochemical studies showed that the prion protein is located predominantly in the synaptic plasma membrane ***(23)*** and, to a lesser extent, in the synaptic vesicle fraction. **Fig. 2** shows a Western blot analysis of PrP^C expression in various synaptic fractions. The enrichment of PrP^C in the synaptic plasma membrane fraction is evident (**Fig. 2A**, lane 4).

2. Electrophysiological Studies

Electrophysiological studies in $Prnp^{0/0}$ mice have been used to identify the function of PrP^C in neurons. Collinge et al. ***(29)*** were the first to describe a change in long-term potentiation (LTP), i.e., a change of synaptic transmission after repetitive stimulation in the $Prnp^{0/0}$ mouse generated by Büeler et al. ***(10)***. This finding was confirmed in a second $Prnp^{0/0}$ mouse generated by Manson et al. ***(30)***. However, Lledo et al. ***(31)*** did not observe LTP changes.

In addition, Collinge et al. ***(29)*** found altered kinetics of the inhibitory postsynaptic currents (IPSCs), i.e., a prolongation of the rise time of $GABA_A$ receptor-mediated IPSCs in hippocampal neurons of $Prnp^{0/0}$ mice. The authors argue that this may be caused by changes in the $GABA_A$ receptor on the

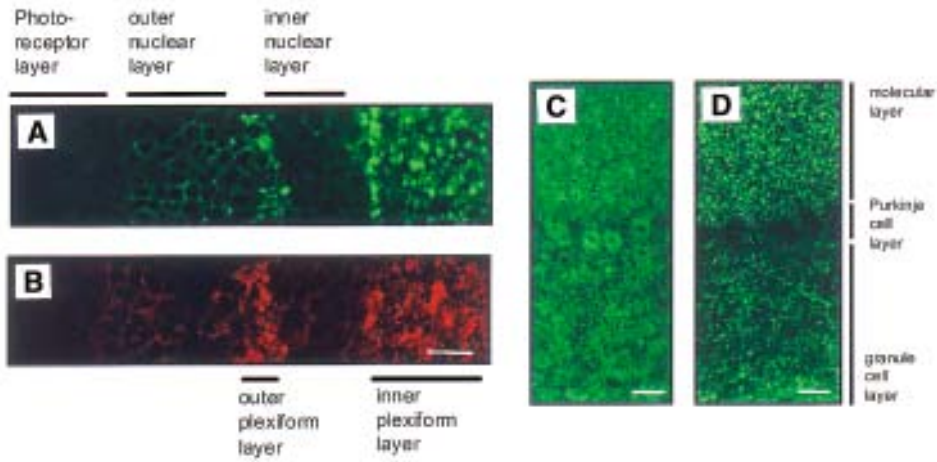

Fig. 1. Synaptic expression pattern of PrP^C in PrP^C-overexpressing transgenic mice. Laser scanning confocal images of PrPc expression in the retina and cerebellar cortex of PrP^C-overexpressing mice. Expression of PrPC (**A**) and synaptophysin (**B**) in Tg20 retina. PrP^C is strongly expressed in the inner and outer plexiform layer, similar to synaptophysin. PrPC expression in Tg35 (**C**) and Tg20 (**D**) cerebellar cortex. Strong PrP^C expression was observed in the molecular and granule cell layers in both transgenic mouse lines. However PrPC expression in Purkinje cells was only observed in Tg35 (**C**).

postsynaptic membrane since a decrease of the amplitude of stimulated inhibitory postsynaptic currents and a shift of the reverse potential of $GABA_A$ receptor-mediated chloride currents were also observed. Lledo et al. ***(31)*** did not confirm this finding for hippocampal neurons of the same knockout line. Also, a more detailed analysis of the kinetics of $GABA_A$-induced currents in outside-out patches from cerebellar Purkinje cells of $Prnp^{0/0}$ mice did not reveal significant deviations from control cells ***(32)***. Moreover, studies on the kinetics of spontaneous inhibitory postsynaptic currents (sIPSCs) in cerebellar Purkinje cells of $Prnp^{0/0}$ mice initially showed significant differences between the rise time of wild-type and that of $Prnp^{0/0}$ Purkinje cells ***(32)***. Further experiments with Purkinje cells of younger animals, with a better voltage clamp (and consequently a more exact estimation of the rise time ***[33]***) showed a significant increase in the rise time, from 1.9 ms in wild-type to 2.81 ms in $Prnp^{0/0}$ mouse Purkinje cells (**Fig. 3D**; P = 0.001). No differences were found in the decay time (**Fig. 3E**). Evidence for the hypothesis that the increased rise time is caused by loss of the PrP^C was found in studies on the rise time in $Prnp^{0/0}$ mice that were Prnp reconstituted (**Fig. 3D**; Tg35; ***[34]***). The IPSC rise time in Purkinje cells of these animals corresponds to the rise time in wildtype ani-

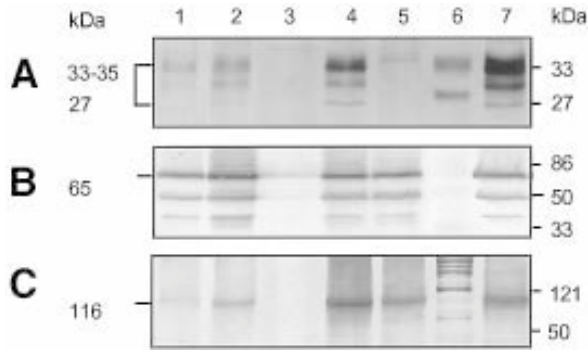

Fig. 2. Enrichment of PrP^C in the synaptic plasma membrane fraction. Preparations of the synaptic plasma membrane fraction and synaptic vesicle fractions from synaptosomes ***(54)***. Equal amounts (100 µg/per lane) of brain homogenate and various subcellular fractions from wild-type (lane 1–4), $Prnp^{0/0}$ (lane 6), and Tg35 (lane 7) mice were investigated in Western blots. The monoclonal antibody 3B5 (**A**); hybridoma supernatant 1:50) ***(55)*** was used to identify PrP^C. A polyclonal antiserum (1:2000) was used to identify the synaptic vesicle protein synaptotagmin (**B**) ***(56)***. The N-methyl-D-aspartate (NMDA) receptor subunit, R1, was shown using the monoclonal antibody, Akp (***C***); (1:2000) ***(55,57)***. Subcellular fractions are designated as follows: lane 1, WT homogenate; lane 2, WT crude synaptic vesicle fraction; lane 3, WT cytosolic synaptic fraction; lane 4, WT synaptic plasma membrane fraction; lane 5, mol w. standards; lane 5 synaptic plasma membrane fraction from $Prnp^{0/0}$ mouse brains. An enrichment of PrP^C (**A**) is noted in the synaptic plasma membrane fraction of wild-type mouse (lane 4), in analogy to the subunit R1 of the NMDA receptor in lane 4 (**C**). In contrast to synaptotagmin, a protein that is predominantly localized to the membranes of synaptic vesicles, PrP^C is not enriched in the synaptic vesicle fraction (lane 2), although it may be found in this location in low concentration.

mals. To clarify the question of whether the increase in rise time in $Prnp^{0/0}$ mice is caused by the loss of PrP^C expression in the presynapse or postsynapse, an additional Tg line, which expresses PrP^C only at the presynapse (Tg20) ***(34)*** was examined. In this line, rise times corresponding to the wildtype were found (**Fig. 3D**). Thus, it appears that the loss of the presynaptic PrP^C expression at the inhibitory synapse is responsible for the prolongation of the rise time of inhibitory postsynaptic currents in $Prnp^{0/0}$ mice.

Independent of the findings at inhibitory synapses, Colling et al. ***(35)*** described an additional electrophysiological phenotype in $Prnp^{0/0}$ mice, i. e. a disturbance of the late afterhyperpolarization current, I_{AHP}. This current is involved in action potential repolarization and therefore influences the frequency of action potentials. Colling et al. ***(35)*** reasoned that the disturbed I_{AHP} in $Prnp^{0/0}$ mice is caused by a decreased conductance of calcium-activated

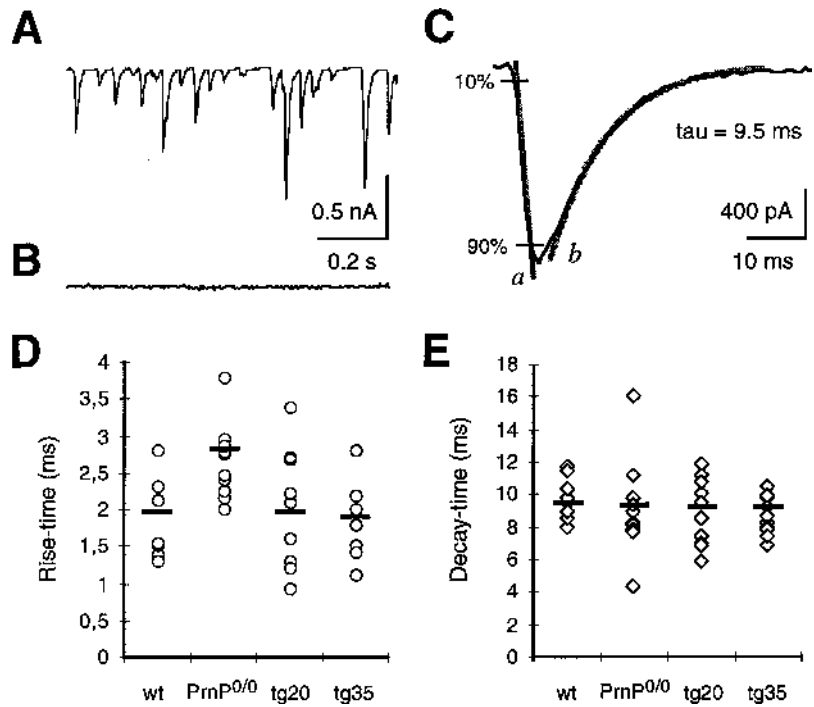

Fig. 3. Presynaptic PrP^C expression modulates the kinetics of inhibitory postsynaptic currents (IPSC). (**A**), Spontaneous IPSCs from a Purkinje cell of a 10-d-old wild-type mouse using the patch-clamp technique, as described ***(32)*** (**B**), Using the effect of 10 μ*M* bicucullin, a γ-aminobutyric acid A ($GABA_A$) receptor blocker, it is shown that the synaptic currents are inhibitory $GABA_A$ receptor-mediated conductances. (**C**), rise time and decay time in wildtype IPSCs. During rise time, there is a linear increase of $GABA_A$ receptor-mediated current from 10 to 90% of the maximum (gray line *a*). The decay time (τ) is calculated from the kinetics of an exponential function (gray line *b*) that shows the best fit to the actual decay of the current. (**D**), Rise time in WT, $Prnp^{0/0}$, Tg20, and Tg35. Shown is the mean of results from each of 10 measurements in Purkinje cells of 9–12d-old animals. Each point corresponds to the rise time of inhibitory postsynaptic currents of a Purkinje cell (mean of the rise time of 20 consecutive IPSCs for each cell). The mean of all measurements is shown as black line. The IPSC rise time is significantly prolonged in $Prnp^{0/0}$ mice compared to wild-type mice ($p = 0.001$, *t*-test according to Welch). No significant differences were found among the rise times of wild-type, Tg20, and Tg35 cells. (**E**), Means of the decay time of IPSCs in wildtype, $Prnp^{0/0}$, Tg20 and Tg35. There are no differences among these mouse lines.

potassium channels, which may be related to a disturbed intracellular calcium homeostasis. This concept is based on findings by Whatley et al. ***(36)*** that indicated an effect of recombinant PrP^C on the intracellular calcium concentration

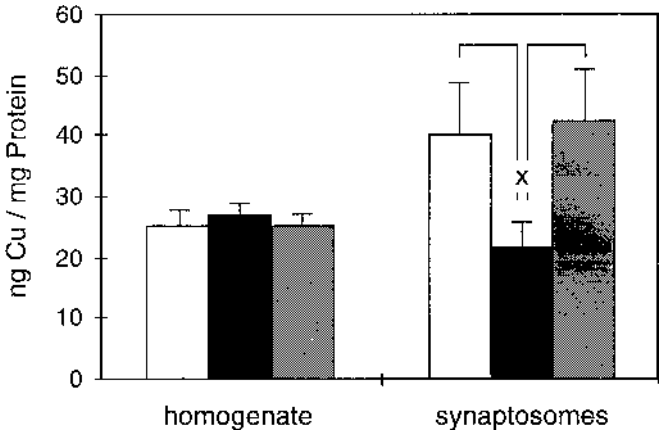

Fig. 4. Copper concentration in synaptosomes correlates with PrP^C expression. The copper concentrations in whole-brain homogenates and synaptosomal fractions from wild-type (open columns), $Prnp^{0/0}$ (black columns), and Tg20 (gray columns) mice were studied by atomic absorption spectroscopy. Shown are the mean and SE of the arithmetic mean of 3–7 preparations from each of five brains of age-matched (2 ± 0.4 mo) female animals of various lines. The copper concentration related to protein concentration in whole-brain homogenates shows no significant differences among wild-type, $Prnp^{0/0}$ and Tg20 mice, but the synaptosomal fraction shows a significant reduction of copper in $Prnp^{0/0}$ mice compared to wildtype and Tg20 mice ($p = 0.03$; *t*-test).

in synaptosomes. Indeed, a study of calcium-activated potassium currents in Purkinje cells of $Prnp^{0/0}$ mice showed a reduced amplitude of these currents (Herms et al., in preparation). Further investigations of transgenic animals which were Prnp reconstituted on the $Prnp^{0/0}$ background (Tg35, Tg20) showed that loss of PrP^C expression in Purkinje cells is responsible for this finding ***(37).*** Thus, a reconstitution of the amplitude of calcium-activated potassium conductances was observed in a transgenic line that shows overexpression of PrP^C in all neurons (Tg35), whereas a transgenic line that overexpresses PrP^C in all neurons but Purkinje cells, showed no reconstitution of the amplitude. The subsequent microfluorometric investigation of the intracellular calcium homeostasis in $Prnp^{0/0}$ mice confirmed that the reduction of calcium-activated potassium currents is probably caused by reduced calcium release from intracellular calcium-sensitive calcium stores ***(37)*** (Herms et al., in preparation).

3. The Role of Copper

The cause of the observed electrophysiological alterations in $Prnp^{0/0}$ mice is not yet known. They may be related to the decreased copper concentration in synaptic membranes of $Prnp^{0/0}$ mice (**Fig. 4**; ***[23]***). The N-terminus of PrP^C has

a highly conserved octapeptide repeat sequence (PHGGGWGQ) x4 ***(38)***, whose possible copper-binding properties were first shown by Hornshaw et al. ***(39,40)*** and later by Miura et al. ***(41)***. The recombinant N-terminus of PrP^C from amino acid 23 to 98 (PrP 23–98) shows a cooperative binding of 5–6 copper ions ***(42)***. Half-maximal cooperative copper binding of PrP23–98 is in the micromolar range (5.9 μM). Further investigations, using synthetic octapeptides ***(43)*** confirmed cooperative copper binding by PrP^C.

The significant decrease of synaptosomal copper concentration in $Prnp^{0/0}$ mouse synaptosomes (**Fig. 4**) may be caused by a decreased reuptake of copper released into the synaptic cleft during synaptic vesicle release, since the difference in the synaptosomal copper concentration between $Prnp^{0/0}$ mice and wildtype mice seems to be too large to be explained solely by the loss of copper bound to PrP^C. In addition, one would then also expect differences in the copper concentration of the crude homogenate in wildtype, Tg20 and $Prnp^{0/0}$ mice (**Fig. 4**). The findings may therefore be explained by a dysregulation of the copper concentration in the brains of $Prnp^{0/0}$ mice caused by loss of PrP^C.

In addition to the decreased synaptosomal copper concentration, a number of further changes were observed that indicated a biological function of copper binding by PrP^C. Thus, significant differences between $Prnp^{0/0}$ mice and wildtype mice were found in inhibitory synaptic transmission in the presence of copper ***(42)***. The application of copper elicited a significant reduction of the mean amplitude of spontaneous inhibitory postsynaptic $GABA_A$ receptor-mediated currents in Purkinje cells of $Prnp^{0/0}$ mice at a concentration of 2 μM Cu^{2+}, whereas this concentration showed no effect on the IPSCs of the wildtype mice. Because it is well known that the $GABA_A$ receptor is functionally disturbed at a concentration of copper in the range of 1 μM ***(44)***, this finding indicates that differences between $Prnp^{0/0}$ and wildtype mice may be caused by missing copper buffering in the synaptic cleft by PrP^C.

It is difficult to verify whether the loss of PrP^C indeed leads to a reduction of the amount of copper located at the synaptic plasma membrane in intact synapses because direct synaptic measurements in vivo are not possible at present. We used an indirect approach to assess the problem of copper binding at the synapse, by studying the effect of hydrogen peroxide on inhibitory synaptic transmission ***(23)***. H_2O_2 is known to alter the probability of synaptic vesicle release by reacting with metal ions, particularly iron and copper at the presynapse, by increasing the presynaptic calcium concentration. By performing patch-clamp measurements on cerebellar slice preparations of wildtype, $Prnp^{0/0}$ and PrP^C reconstituted transgenic mice, we observed the effect of 0.01% H_2O_2 on the frequency of spontaneous IPSCs in Purkinje cells correlate with the amount of PrP^C expressed in the presynaptic neuron (**Fig. 5**). This indicates that the amount of copper at the synapse may indeed be PrP^C-related.

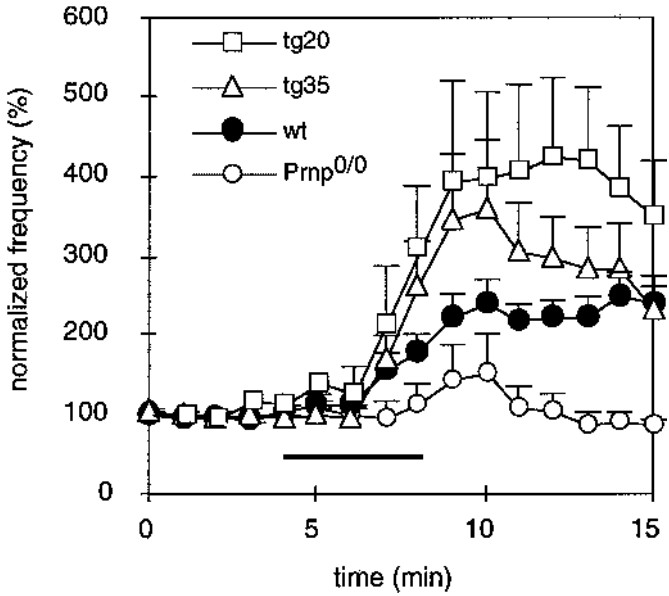

Fig. 5. Enhancement of inhibitory synaptic activity by hydrogen peroxide is related to the amount of PrP^C at the presynaptic plasma membrane. Effect of 0.01% H_2O_2 on the frequency of inhibitory postsynaptic currents in the different mouse lines. Each point represents the mean ± SEM sIPSC frequency in 1-min intervals normalized to the values before H_2O_2 application of wild-type ($n = 14$), $Prnp^{0/0}$ ($n = 21$), Tg35 ($n = 15$) and Tg20 ($n = 4$) mouse Purkinje cells. The bar indicates the time during which H_2O_2 was applied. The application of H_2O_2 led to a marked enhancement of synaptic activity in wild-type mice, there is no comparable effect in $Prnp^{0/0}$ mice. In transgenic mice that overexpress PrP^C on a $Prnp^{0/0}$ background in all neurons (Tg35), the sIPSC frequency increase after H_2O_2 application is rescued. Also, PrP^C-reconstituted mice, which express PrP^C in cerebellar interneurons, but not in Purkinje cells (Tg20), show a rescue, indicating that the presynaptic PrP^C expression is important for the rescue of the H_2O_2 effect on IPSC frequency.

It remains to be shown whether buffering of copper released during synaptic vesicle release, which prevents or minimizes unspecific binding of copper to other proteins, is the primary function of PrP^C (**Fig. 6**). Alternatively, the binding of copper to PrP^C may primarily serve the reuptake of copper into the presynapse by endocytosis of PrP^C ***(45,46)*** or may be of structural importance for the N-terminus of PrP^C ***(47)***.

The hypothesis of a functional re-uptake of copper in the synaptic cleft by the prion protein (**Fig. 6**) may explain electrophysiological findings in $Prnp^{0/0}$ mice, which, on first glance, seem contradictory. A slight increase of extracellular copper concentration, caused by decreased or missing copper buffering in

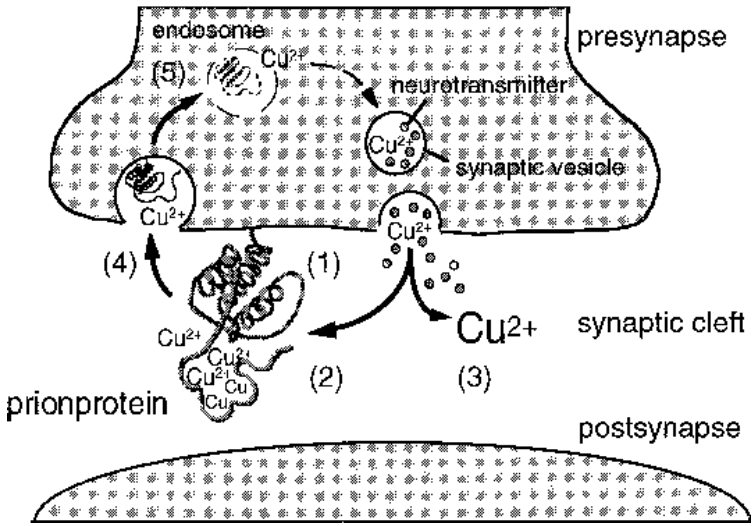

Fig. 6. Hypothetical model showing a possible function of copper binding by PrP^C at the synaptic plasma membrane. The prion protein is attached to the presynaptic plasma membrane (1) ***(23)***, where its N-terminal moiety (2) binds free copper that is released into the synaptic cleft with synaptic vesicle release (3) ***(58,59)***. There is an endocytotic uptake of PrP^C into the presynapse (4) ***(45,46)*** where PrP^C-bound copper is released, possibly induced by endosomal pH changes (5) ***(43)***. Thus PrP^C serves to keep the copper concentration in the presynaptic cytosol and the synaptic cleft constant despite copper losses during synaptic vesicle release (3).

the synaptic cleft in $Prnp^{0/0}$ mice, may cause a decrease in the conductance of voltage-activated calcium channels and a change in the kinetics of the $GABA_A$ receptor. Thus, the conductance of the $GABA_A$ receptor and voltage-activated calcium channels, which modulate intracellular calcium homeostasis is clearly disturbed by copper concentrations of 1–10 μ*M* ***(44,48)***. This would explain the alteration of the intracellular calcium homeostasis in $Prnp^{0/0}$ mice, changes in the conductance of calcium-related ion currents, and changes in $GABA_A$ receptor-related inhibitory postsynaptic currents observed under certain conditions. Reduced LTP in $Prnp^{0/0}$ mice may be explained by this hypothesis, as well. As shown by Doreulee et al. ***(49)***, LTP is blocked by concentrations of free copper as low as 1 μM. Changes in the circadian rhythm observed by Tobler et al. ***(12,13)*** in $Prnp^{0/0}$ mice could be related to a disturbed copper uptake and a decreased activity of copper-dependent enzymes, since the synthesis of melatonin, which is important in the regulation of circadian rhythms ***(50)***, is regulated by the copper-dependent enzyme monamine oxidase ***(51)***. Also, the activity of two other copper-dependent enzymes, the Cu/Zn superox-

ide dismutase and the glutathione reductase have been found to be altered in $PrP^{0/0}$ mice ***(52,53)***.

4. Conclusion

In summary, our studies have shown that PrP^C binds copper cooperatively and with high affinity. In the brain highest concentrations of PrP^C are found at synapses. Synaptosomes of $Prnp^{0/0}$ mice demonstrate a strong reduction of copper concentration. Copper binding by PrP^C in the synaptic cleft has a significant influence on synaptic transmission. It remains to be shown whether additional phenotypes observed in $Prnp^{0/0}$ mice result from decreased copper binding or from a disturbance of copper distribution in the absence of PrP^C.

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3

A Function for the Prion Protein?

David R. Brown and Ian M. Jones

1. Introduction

Protein function is often observed directly following protein isolation, or is deduced by loss of function following gene knockout or by analogy with proteins of known function and similar amino acid sequence. None of these is true in the case of prion proteins because aside from the association with the pathogenesis of the spongiform encaphalopathies, no single obvious function has been described for these molecules until recently. The first two chapters in this volume (*see* **refs. *1–3***), concentrated on the characterization of the infectious agent, and led to the introduction of the term "prion" in 1982 ***(4)***. But it was not until the positive association of the infectious agent, PrP^{Sc}, with a normal host gene locus, *prnp,* that real opportunities to consider protein function in relation to the disease phenotype arose ***(5)***. The identification of the prion gene on chromosome 2 of the mouse (chromosome 20 in the human) ***(6)***, and the determination of its sequence ***(7)***, led to the translation of the encoded protein and speculation concerning its function.

2. Prion Sequences

Translation of the original DNA sequences of the mouse and hamster prion genes described the structural features of the protein. Prion protein is 254 amino acids long (253 in the human) and has an unusual structure, which was unique when first described **Figure 1**. The coding region for the mature protein is preceded by a 22 amino acid signal peptide and followed by a sequence of 23 amino acids that is removed following expression and replaced by a glycosyl phosphatidylinositol (GPI) tail at serine 231. These features mark the protein

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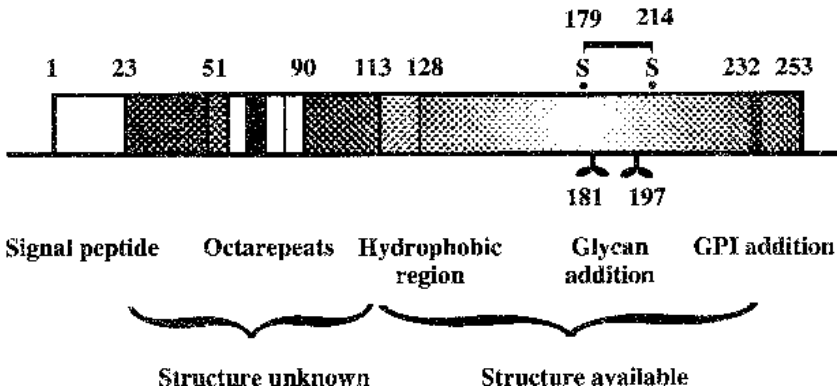

Fig. 1. Schematic representation of the prion protein amino acid sequence. Details are taken from the mouse sequence, but the overall features are shared by all prion proteins. Numbers along the scheme indicate the amino acid positions of each feature and a correlation with the known three-dimensional structures is given below. The position of the single disulphide bond and the glycosylation sites are indicated.

for expression on the cell surface and make it conceivable that PrP^c may be grouped with other GPI anchored proteins that are commonly associated with cell to cell signalling, adhesion or cellular defense ***(8)***. Secondary structure predictions, even prior to the derivation of a three-dimensional structure, suggested that the carboxy terminal domain was likely to adopt a distinct fold mostly made up of α-helices, but that the N-terminal domain lacked clear structural features. The carboxyl domain also contains a single disulphide bond between residues 179 and 214 and two potential N-linked glycosylation sites at residues 181 and 197. Although lacking in unambiguous secondary structure predication, the amino terminal domain of PrP^c has a unique sequence of eight amino acids, rich in glycine and proline, that is repeated 4× between residues 51 and 91, and is separated from the carboxyl half by a stretch of hydrophobic residues centered on residue 120. The PrP^c sequence is unique, making impossible the prediction of a function based on linear or secondary structure comparisons.

The sequence of PrP^c from a number of other species has since been determined. They show a remarkable degree of conservation at the amino acid level (**Fig 2**), and the same set of unique structural features. The sequence of avian PrP shows a greater number of repeats, with fewer amino acids in each ***(9)***, a feature shared with the recently determined reptilian (turtle) PrP ***(10)***. All other features of the protein have confirmed, however, an unprecedented homology among so diverse a set of organisms. It seems likely from these data that prion proteins with essentially the same structure are present in all verte-

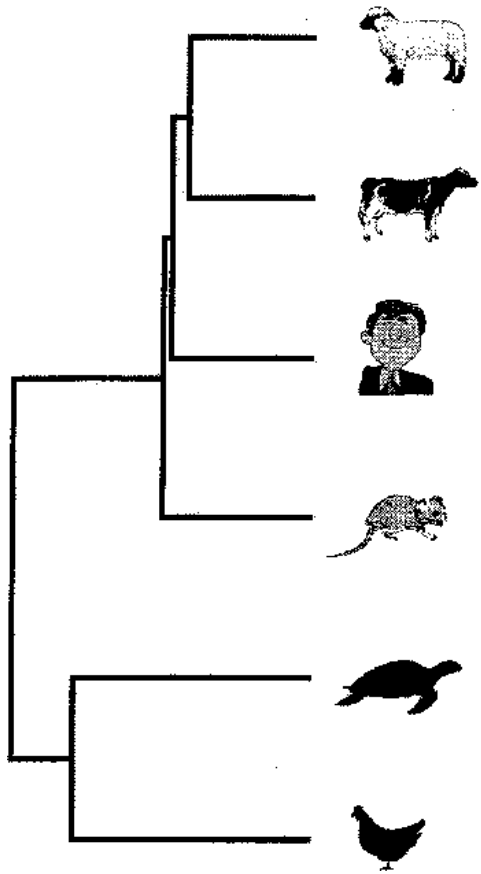

Fig. 2. Alignment of the sheep, cattle, human, mouse, turtle and chicken prion amino acid sequences showing the overall conservation of sequence and split between the mammalian and reptilian/avian sequences.

brates, a fact that may signify an important role for the encoded protein and a tight conservation of sequence throughout evolution. The original sequence of PrP^c suggested that the molecule had features similar to some proteins with a tendency to aggregate ***(7)***, and that this may explain the predisposition to amyloid formation. More recently however, many proteins have been shown to be capable of forming amyloid, under the correct conditions, so that the requirement for particular sequence motifs is uncertain ***(11)***.

A recent investigation ***(12)*** has identified the first prion protein-like gene *(prnd)* in the mouse. *Prnd* expresses a short protein with homology to the C-terminal domain of PrP^c. The protein, termed Doppel, is not normally

expressed in adult mice, but is expressed in two strains of $Prnp^{o/o}$ mice. These mice differ from others, in that they develop late-onset neuropathological changes including Purkinje cell degeneration ***(13)***. Speculation concerning the mechanism of Doppel expression has centered on the possibility that the genetic manipulation used to create the two strains of $Prnp^{o/o}$ mice caused a deletion of specific inhibitor sequences between the *prnp* and *prnd* genes resulting in the *prnd* gene being transcribed by the *prnp* promoter. Despite the similarities between the two proteins, Doppel lacks most of the more highly conserved regions, including the hydrophic core region and the octameric repeats. It is unlikely, therefore, that Doppel and PrP^c have a common function although interference of either by the other remains a possibility.

3. Prion Structure

It is not unusual for proteins to exhibit similar folds in the absence of significant sequence homology (e.g., those shared by HIV matrix antigen and interferon γ ***[14]***). Thus, although direct alignment of prion sequences with those in the databases failed to identify matches, homology based on the tertiary structure of the protein could be instructive. The solution of the structure for the carboxyl domains of mouse and hamster PrP^c, obtained by nuclear magnetic resonance spectroscopy ***(15,16)*** has, however, failed to suggest a role for the molecule. The C-domain is well ordered, containing three α-helices and a short section of antiparallel β-sheet (**Fig 3**). The GPI anchor occurs at the end of the final helix and suggests an orientation of the molecule with respect to the cell membrane (**Fig 3**). The two longest helices are held together by the single di-sulphide bond. Recent evidence suggests the carboxyl domain folds very rapidly, and is mostly unaffected by variation in pH or temperature ***(17)***. The N-terminal domain, by contrast, which includes the octameric repeats, has no defined structure. The lack of distinct secondary and tertiary structure to the N-terminal domain of PrP^c coupled with the unusual structure of the octarepeats suggest that ligand binding in this region may be necessary for the adoption of a stable tertiary structure.

Using peptides representing only the octarepeats Hornshaw et al. showed that copper, as Cu^{++}, was bound by both the mouse and chicken sequences ***(18)***. Equilibrium dialysis experiments, first using a recombinant fragment of PrP^c equivalent to the N-terminal region to amino acid 98, and later with full length recombinant or wild-type PrP^c, has since confirmed that mouse PrP^c binds several atoms of copper ***(19–21)***. Copper binding was not observed however following expression and purification of full length chicken prion protein ***(22)***. Moreover, further studies on peptides representing the octarepeat region have not yet allowed an unambiguous mechanism of copper binding to be formulated. Viles et al., using a variety of spectroscopic techniques, concluded

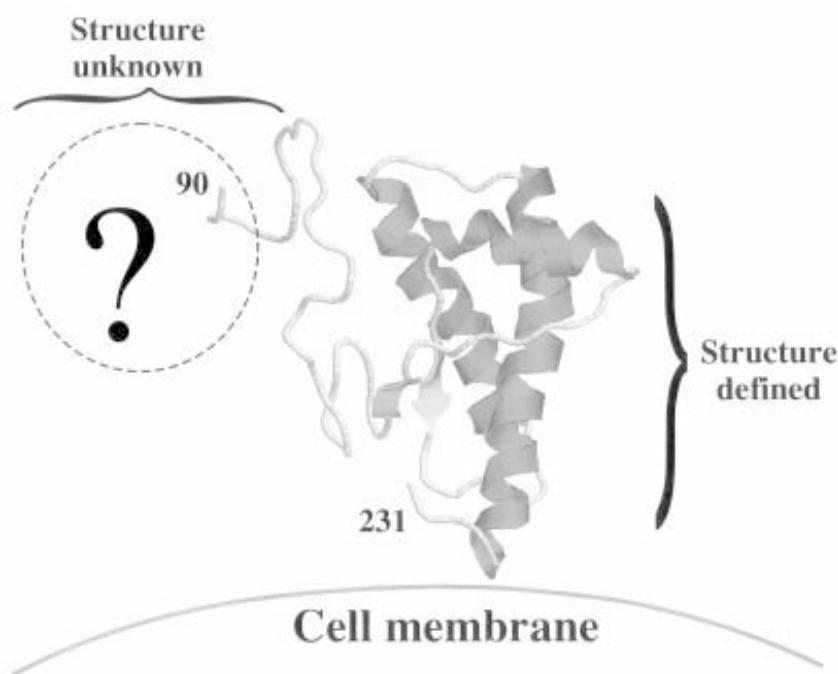

Fig. 3. The structure of the Golden hamster prion protein taken from the published work of James et al., (*16*). The 142-amino acid protein extends from position Gly_{90} to Ser_{231} and contains three γ-helices and two short sections of β-sheet. A structure is unavailable for the region prior to amino acid 90. The addition of the GPI tail to Ser_{231} suggests the orientation to the cell membrane as shown, but this is not proven.

that copper was co-ordinated by the histidine residues of each octarepeat in a tetrad planer arrangement reminiscent of Cu/Zn superoxide dismutase ***(23)***. By contrast, Miura et al., concluded that the form of contact with copper depended heavily on the pH of the interaction, and suggested that, at neutral and basic pH, each copper ion was bound in an intrachain configuration by two adjacent glycine and one histidine residues. Under weakly acid conditions, however, copper binding changed to an interchain configuration, with implications for the formation of prion aggregates ***(24)***. Similar uncertainty surrounds the redox state of the bound copper. Ruiz et al., using a copper chelator, bathocuproine disulfonate (BC), suggested that the bound copper was present in reduced form, and that the tryptophan residues present in each repeat sequence were the likely redox acceptor ***(25)***. Shiraishi et al., also used BC to measure the redox state of bound copper but concluded that the metal was present in the nonreduced form ***(26)***. They suggested that sequestering of the metal as the divalent cation pre-

vented copper induced generation of reactive oxygen implicating PrP^c as a protective metal chelator molecule. Wong et al. reported extensive methionine oxidation in preparations of recombinant mouse and chicken prion proteins following refolding in the presence of copper suggesting that the copper ion is redox active when present within the full length protein ***(27)***. Other copper binding proteins involved in the transport of copper across mammalian cell membranes are thought to bind copper in the Cu^+, rather than the Cu^{++} form ***(28)***.

If copper is a natural ligand of PrP^c, then it is a reasonable premise that prion protein function depends on its acquisition, or that imbalances in copper level, and the consequences of it, would go hand in hand with the presence or absence of PrP^c.

4. Resistance to Oxidative Stress

The experimental production of prion protein knockout mice allowed an investigation of the role of PrP^c in prion disease. $Prnp^{o/o}$ mice do not express the product of *prnp,* yet remain healthy, suggesting that loss of PrP expression does not directly result in disease. A role for the protein is clear, however, from the observation that $Prnp^{o/o}$ mice cannot be infected with the scrapie agent. Although susceptibility to infection is an identifiable phenotype for PrP^c, it seems nonsensical to suppose that this is its only cellular role, given the evolutionary conservation apparent in PrP sequences.

Analysis of the neurotoxicity of both PrP^{Sc} and the neurotoxic peptide, PrP106–126, in cell culture experiments using cells derived from $Prnp^{o/o}$ mice indicated that PrP^c expression is also necessary for the observed toxicity ***(29,30)***. This was later confirmed in the mouse model of scrapie. Following transplantation of PrP^c expressing brain tissue into $Prnp^{o/o}$ mouse brains, and infection of the transplanted mice with the scrapie agent, PrP^{Sc} accumulated in PrP^c-expressing tissue. The surrounding tissue was free of neurodegeneration, indicating that PrP^{Sc} was not toxic to PrP^c-deficient neurons ***(31)***.

Further analysis of PrP106-126 toxicity indicated that the peptide kills neurones in culture by causing toxic radical release from microglia and by inducing a reduced resistance to those radicals in neurones. PrP106-126 could only reduce the resistance to oxidative stress in neuron cultures that express PrP^c ***(32,33)***. The reactive oxygen species produced by wild-type microglia in the presence of PrP106-126 were insufficient to kill neurones that did not express PrP^c ***(32)***, or neurones not exposed to the peptide (D. R. Brown, unpublished data).

Although the brains of $Prnp^{o/o}$ mice (lacking Doppel expression) are normal ***(34)***, neuronal cultures produced from neonatal $Prnp^{o/o}$ mice show greater sensitivity to culture conditions, and died at a faster rate than wild-type neurons

(33). A similar observation has been made for cell lines generated from other $Prnp^{o/o}$ mice ***(35)***. The decreased viability of $Prnp^{o/o}$ neurons in culture was found to result from increased sensitivity to oxidative stress ***(33)***. Superoxide, generated enzymatically in the cultures, also killed more $Prnp^{o/o}$ neurons than wild-type neurons ***(33)***. Using PC12 cells, increased resistance to oxidative stress was found to correlate with increased PrP^c expression ***(36)***. This also correlated with increased sensitivity to the toxic effects of PrP106-126. There is, therefore, a strong parallel between the in vitro phenotype of PrP^c-deficient cells and the phenotype induced by PrP106–126, at least in terms of resistance to oxidative stress.

Studies on the activity of the antioxidant, cytosolic enzyme, Cu/Zn superoxide dismutase SOD-1 also support the idea that PrP106–126 may induce a PrP^c-deficient phenotype in neurons. PrP106–126 treatment induced decreased activity of SOD-1 in cultured cerebellar cells ***(33)***. Studies of the brains of two strains of $Prnp^{o/o}$ mice indicated that these mice have reduced SOD-1 activity in vivo without treatment ***(19,33)***. The reduction in SOD-1 activity was not caused by decreased expression of protein or transcription of messenger RNA, but was likely to have resulted from decreased incorporation of copper, necessary for activity, into the SOD-1 molecule ***(37)***. Furthermore, mice expressing higher levels of PrP^c than wild-type mice had correspondingly higher SOD-1 activity in their brains.

Further study of cultured cells provided other examples of diminished cellular resistance to oxidative stress in PrP^c-deficient neurons. Although the activity of glutathione peroxidase and catalase appear to be unaltered in neuronal cultures from $Prnp^{o/o}$ mice, there is evidence for altered glutathione metabolism resulting from changes in the activity of glutathione-S-transferase (GST) ***(38)***. A similar result was obtained for cells treated with PrP106–126, which also diminished the activity of GST, as well as depleting cells of the reduced form of glutathione ***(39)***.

Despite a wealth of evidence from cell culture experiments which suggests that PrP^c expression is linked to resistance to oxidative stress it is important to note that there is, as yet, no evidence that this is a significant role for PrP^c in vivo.

5. Copper Metabolism

Experiments with PC12 cells indicated that those expressing high levels of PrP^c were more resistant to oxidative stress. Additionally, the same cells were found to be more resistant to copper toxicity ***(40)***. Furthermore, a PC12 cell line selected for its resistance to copper toxicity, was also more resistant to oxidative stress and showed higher levels of PrP^c-expression than standard PC12 cells ***(40)***.

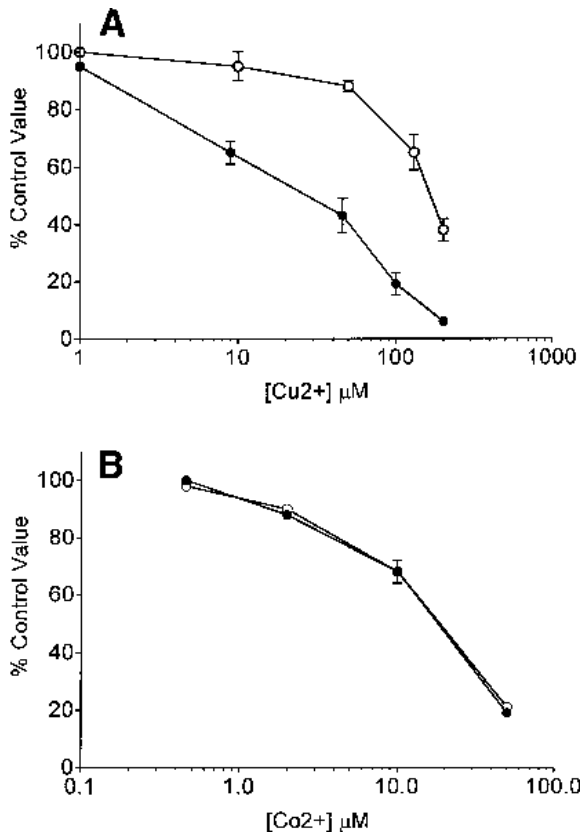

Fig. 4. Toxicity of copper and cobalt to cultures of 6-d-old cerebellar cells. The cells were treated for 2 d with $CuCl_2$ or $CoCl_2$. They were then assayed for relative cell survival, using a standard MTT assay. Control = 100% survival o-o wt; ●-● $Prnp^{o/o}$.

Cultures of primary neurons or astrocytes from $Prnp^{o/o}$ mice were also found to be more sensitive to the toxicity of copper ***(41)***. Cultures from mice overexpressing PrP^c were more resistant to copper toxicity than wild-type cells. Other divalent cations were not more toxic to $Prnp^{o/o}$ cerebellar neurones than to wild-type neurons suggesting PrP^c-expression selectively protects against copper toxicity (**Fig 4**).

Peptides based on the octarepeat sequence bind copper (*see* **Subheading 3**.) ***(42,43)***. When a 32-amino acid peptide encoding this sequence was added to cultures of cerebellar neurons, it protected against the toxicity of copper. This effect was strongest on $Prnp^{o/o}$ cerebellar neurons ***(41)***. Additionally, this peptide protected against superoxide toxicity. Copper can convert superoxide to other toxic substances, and the mechanism of peptide action could have been

through copper binding and prevention of these reactions. Depletion of the cellular ability to bind copper has been shown to increase cellular sensitivity to copper toxicity ***(44)***. An antibody that binds near the octameric region was found to specifically enhance the toxicity of copper ***(41)*** possibly through prevention of copper binding to the PrP^c expressed by wild-type neurons. Together, these results suggest that PrP^c can act as a copper chelator.

PrP^c immunoprecipitated from mouse brain contains large amounts of copper but no other divalent cation ***(21)***. Cultured cells from wild-type cells contain more copper than those from $Prnp^{o/o}$ mice ***(19)***. The difference between wild-type and $Prnp^{o/o}$ cerebellar cell membrane fractions in terms of copper content can be abolished by treatment with an enzyme that cleaves GPI-anchored proteins from cells, suggesting that the difference in copper content of wild-type cells result from binding of copper by one or more GPI anchored proteins, such as PrP^c ***(45)***. The synaptosomal fraction of mouse brain also contains large amounts of copper, much higher than in similar preparations from $Prnp^{o/o}$ mice (**Fig 5**); ***(19)***. It is clear that PrP^c is highly expressed at the synapse ***(46)***, leading to the conclusion that PrP^c binds copper both in vitro and in vivo.

The brain has high levels of copper, second only to the liver. The brain shows sensitivity to imbalances in copper levels. In Wilson's disease and Menke's disease, mutations in P type adenosine triphosphatases (ATPases) alter copper metabolism (for reviews, *see* ***refs***. ***[47–49]***). In Menke's disease there is failure of copper transport from the intestine which leads to copper deficiency ***(50)*** and the inability of the brain to develop normally. Copper deficiency may lead to neurodegeneration ***(51)***. In Wilson's disease, mutations in a P type ATPase ***(52)*** found mostly in the liver, lead to an increase in deposition of copper in the brain and kidneys ***(53)***. This is probably due to failure of the Wilson type ATPase to transport copper across the canalicular membrane of the liver into the bile ***(54)***. In addition, release of the main serum transporter of copper, ceruloplasmin, is impaired. This is probably caused by the failure of the Wilson-type ATPase to donate copper to the necessary proteins in the excretory pathway. The accumulation of copper in the brain subsequently leads to neurodegeneration. The sensitivity of the brain to copper suggests that control of copper uptake and detection of abnormal levels of extracellular copper are important. Copper is necessary to the brain in terms of the activity of molecules such as SOD, cytochrome C and tyrosinase, and also for synaptic transmission. However, the exact mechanism of copper uptake by the brain remains unclear.

High copper content has been previously localized to the secretory apparatus of neuronal terminals ***(55,56)*** from where it is released upon depolarization ***(57)***. In this situation, there is a high local accumulation of copper that must be dealt with rapidly after the transmission event. The copper that is released at

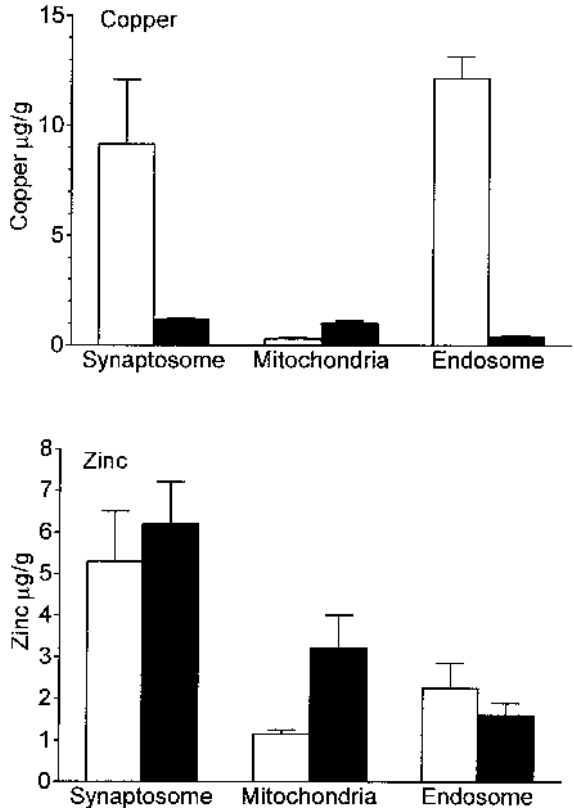

Fig. 5. Analysis of subcellular fractions of cells from 10-d-old wild-type (open bars) and PrP knockout mice (closed bars) for the levels of two divalent metal cations, copper and zinc. The mitochondrial data are unpublished work of DRB.

these sites is initially taken up by a high-affinity copper binding process ***(58)***. The presence of copper in the synaptic cleft is important for the modulation of many kinds of synapses. High copper reduces γ-aminobutyric acid (GABA) and glutamate uptake ***(59)*** and reduces GABA-induced currents ***(60)***. However, copper enhances dopamine uptake ***(61)***. Copper also regulates the distribution of muscarinic cholinergic receptors, enhancing uptake at low concentration, and inhibiting at high concentrations ***(62,63)***. Copper-deficient mice show increased levels of GABA receptors and muscarinic cholinergic receptors ***(64)***. Copper can inhibit transmission at N-methyl-D-aspartate (NMDA) receptors, and shows a higher affinity for NMDA receptors to which the agonist has already bound ***(65)***. Collinge et al. ***(66)*** found defects in synaptic activity of PrP^c-deficient mice, which included reduced long-term potentiation, which is dependent on NMDA receptor activity, and also a reduction in

GABA-type inhibitor currents ***(66)***. Therefore, it is possible that PrP^c may assist synaptic transmission by preventing the deleterious effects of copper release at the synapse.

6. A Molecular Function for PrP^c

Copper is an abundant cation with an ability to capture electrons. Metabolic control of copper is essential both for prevention of harmful effects, such as generation of oxidative damage, by its ability to generate reactive oxygen species in the presence of water, and also because it can be utilized to control electron transfer such as occurs in respiration or dismutation of superoxide ***(67)***. Copper binding proteins are therefore essential for normal cellular metabolism. Because PrP^c is a membrane-associated protein, it may function similarly to other membrane-associated copper binding proteins which eliminates a role for PrP^c purely as a storage protein, such as the copper binding metallothionens. Similarly, although copper sequestration may be an advantage of PrP^c expression, the abundance of extracellular copper transporting proteins, such as albumin and transferrin, eliminate this as a likely sole function.

Three classes of copper-binding proteins are membrane-associated ***(68)***. First, oxidases such as cytochrome C, are mostly associated with the mitochondria, and are involved in respiration, an unlikely function for PrP^c. Second, extracellular SOD exists in a membrane-bound form, and, as previously stated, cells from $Prnp^{o/o}$ mice have reduced resistance to oxidative stress, and have reduced levels of SOD activity ***(33)***. The changes in phenotype were originally considered to result from decreased incorporation of copper into the cytosolic SOD. PrP^c is a small molecule and it seems likely that any substrate molecule would also be small. Investigation of possible SOD activity of PrP^c, therefore, has some validity. Third, some membrane-associated copper binding proteins are involved in copper translocation. Use of Cu^{67} has identified two possible copper-uptake mechanisms into neuronal tissue with high and low affinities ***(69)***. However, the nature of the proteins responsible for this has not been identified.

6.1. Copper Uptake and Release

Little or no free copper exists in the brain. Copper is mostly present on transport proteins or as chelates with other compounds, such as peptides or amino-acids. Accordingly, uptake of copper into neurons is greatly enhanced when the copper is provided in the form of a chelate. The fate of added copper was followed using radioactive copper with Cu^{67} because its half life is greater than Cu^{64} and because Cu^{64} often contains a higher percentage of contaminants. Three strains of mice were used for these studies: mice overexpressing PrP^c, wild-type mice, and PrP^c-deficient mice. Uptake of nonchelated Cu^{67} was

identical for all three strains of mice. However, Cu^{67} provided as a histidine chelate was taken up at a rate that could be related to the level of expression of PrP^c ***(70)***. Membrane fractions from cerebellar cells overexpressing PrP^c showed the highest binding of Cu^{67} whilst the lowest binding was by PrP^c-deficient cells. The rate of entry of Cu^{67} into the cell was assessed in greater detail and kinetic parameters determined. Values for V_{max} increased with increased expression of PrP^c. On the other hand values of K_m (in the n*M* range) were not greatly different, the only significant difference being between overexpressing and PrP^c-deficient cells ***(70)***. These differences in Cu^{67} uptake are consistent with the idea that there is an increase in the number of Cu^{67}- binding sites between the three strains, which may be related to the level of PrP^c expression.

Immunoprecipitation of the cytosolic enzyme, SOD-1, from cells loaded with Cu^{67} indicated that Cu^{67}, could be incorporated into SOD-1 in proportion to the level of PrP^c expressed by the cells ***(37)***. This suggests that Cu^{67} can be incorporated into cellular proteins when taken up in association with PrP^c.

The highest level of PrP^c expression is at the synapse (above), and, because copper is released during quantal release, it is possible that PrP^c may have an important role in regulating copper concentrations there. Cells loaded with Cu^{67} for 30 min were allowed to release copper spontaneously over a period of 1 h, and reached a plateau after 30 min, when the amount of copper was stable ***(70)***. Cells prepared in this way were then treated with veratridine, which is a depolarizing agent that induces release similar to synaptic release. Veratridine induced release of copper from cells at a level that could be related to the expression of PrP^c. Cells expressing no PrP^c released almost no copper when induced by veratridine; PrP^c-overexpressing cells released more copper than wild-type cells ***(70)***.

Electrophysiological experiments indicate that copper applied to cerebellar slices inhibited the amplitude and frequence of inhibitory currents measured on Purkinje cells of PrP^c-deficient cells but not on wild-type cells ***(19)***. This suggests that some form of protection against copper is missing at PrP^c-deficient synapses, and that such protection could be mediated by PrP^c. These results suggest that PrP^c expression alters a number of aspects of copper metabolism including copper uptake, copper utilisation, synaptic release and may aid in protective sequestration of copper.

6.2 A Synaptic SOD

Sources of recombinant PrP protein ($rPrP^c$) from bacterial expression systems have allowed direct assessment of the ability of PrP to bind and use copper ***(21)***. After purification of $rPrP^c$ and subsequent refolding of the protein from urea, $rPrP^c$ was found to be both proteinase sensitive and to possess a secondary structure similar to that found by other researchers ***(71)***.

Refolding of the protein in the presence of copper led to copper binding to the octameric repeat region as shown by the failure of a specific deletion mutant lacking residues 59–91 to bind the same level of copper ***(21)***. Direct measurement of the copper bound suggested that four copper ions bound per complete octarepeat region confirmed the measurements made in earlier work ***(23)***. Copper bound to the Histidine repeat region at the C-terminus of the protein, added to facilitate purification, could be eliminated by removal of the tail by partial trypsin digestion ***(21)***. The solubility of the protein was increased when the protein was refolded in the presence of copper, suggesting that it adopted a more compact structure in keeping with the increase in secondary structure measured for octarepeat peptides following copper binding ***(43)***.

The $rPrP^c$ protein with and without specifically bound copper was also quantitatively assayed for SOD activity. These assays indicated that $rPrP^c$ (from both chicken and mouse) could catalyze superoxide dismutation at a rate equivalent to one tenth that of SOD-1 (based on protein concentration), one of the most potent enzymes known which catalyzes superoxide dismutation at around 100,000× the spontaneous rate ***(72)***. Thus, $rPrP^c$ has SOD-like activity in vitro, a finding that was confirmed for native protein immunoprecipitated from mouse brain ***(21)***.

Stringent controls were carried out to ensure that the activity measured was a form of enzymatic catalysis, and was not caused by simple Fenton chemistry arising from the ligating of copper to the protein. Ethylenediamine tetracetic acid does not inhibit the SOD-like activity of $rPrP^c$. Deletion of the specific octameric repeat region of the protein abolishes the activity, even when copper is bound to the uncleaved His-tag ***(21)***. A peptide based on the octameric repeat region, and with copper bound to it, has no SOD-like activity. Similarly, if $rPrP^c$ is refolded without copper and copper is added afterwards, the copper/$rPrP^c$ mixture does not show the SOD-like activity of $rPrP^c$ refolded with copper ***(21)***. Therefore, the observed catalytic activity is not the result of presence of copper alone, suggesting that a catalytic site is required for the activity. Deletions of regions in the C-terminus of the protein abolish the activity, but do not prevent copper binding to the octarepeat region (D. R. Brown, unpublished observations). Further work is required to delineate the active site, but it is clear that regions outside the octameric repeat region are required for the SOD-like activity. There was a high degree of methionine oxidation associated with copper-refolded $rPrP^c$ which, as most methionines are in the C terminal domain, indicated the involvement of the whole molecule in a redox-based reaction ***(27)***. This is characteristic of anti-oxidant copper binding enzymes, such as SOD-1 ***(73)***.

Currently, there are three accepted mammalian SODs ***(74)***. The first of these, Cu/Zn superoxide dismutase (SOD-*1*), is found only in the cytosol. It consists

of a dimer of two identical subunits, one of which binds copper and the other zinc. Manganese superoxide dismutase (MnSOD, or SOD-2) is found in the mitochondria and binds manganese. These two enzymes are found in all cells at varying concentrations, and often show increased expression or activity in the presence of oxidative stress. Similarly, PrP^c expression increases when PC12 cells are grown in the presence of oxidative stress ***(36)***. The third mammalian SOD is known as extracellular superoxide dismutase (EC-SOD or SOD-3) ***(75)***. It exists in three different isoforms, and, like PrP^c, binds four copper atoms, and is either released into the extracellular environment or is bound to the cell surface. However, the expression of SOD-3 is very low in the brain ***(76)***. PrP^c, on the other hand, has its highest expression in the brain, and is also highly expressed at neuromuscular junctions ***(77)***. In the brain, the expression of PrP^c is highest at synapses, and it is conceivable that it may act as a synaptic SOD that may even be released during transmission. Superoxide is known to inhibit synaptic transmission and the presence of SOD activity at the synapse may have protective effects.

The expression of PrP^c is not restricted to neurons or even to the synapse. In neurons, mostly one glycoform of PrP^c is transported from the soma to the axonal terminals ***(78)***. Astrocytes ***(79,80),*** microglia ***(81)***, leukocytes ***(82)*** and cells of other nonneuronal tissues ***(83,84)*** also express PrP^c. Therefore, its activity as an SOD, although of less direct consequence, may not be limited to the synapse or the brain.

Clearly, the identification of SOD activity associated with purified PrP^c requires further verification. If confirmed, however, a defined enzymatic function for PrP^c has broad implications for understanding prion disease. The changes in secondary structure, following conversion of PrP^c to PrP^{Sc}, mean that it is unlikely that PrP^{Sc} would retain SOD activity. As PrP^c levels are depleted during the accumulation of PrP^{Sc}, oxidative damage to the expressing cells would ensue. Alternatively, if PrP^{Sc} retained some capacity to bind copper, Fenton chemistry, unchecked by the action of PrP^c could inducing oxidative damage to neurons in the vicinity of PrP^{Sc} deposits. Alternatively, deposition of chelated copper within PrP^{Sc} could cause local copper depletion and induce reduced resistance to oxidative stress, an effect that has already been observed in culture for the neurotoxic PrP peptide, PrP106–126.

The finding that PrP^c can exhibit a specific antioxidant activity which requires copper links together the physical measurement of copper binding by PrP^c with the biology of PrP^c loss. Lack of PrP^c expression results in reduced resistance to oxidative stress, and results in a depletion of copper found at the synapse. This observation is not just true for neurons. Astrocytes deficient in PrP^c expression are also more sensitive to oxidative stress ***(85)***, and cell mem-

brane fractions from such cells have lower copper content (D. R. Brown, unpublished observations).

Together, these results indicate a role for PrP^c consistent with its distribution and known biochemical properties to date, and strongly suggest the possibility that PrP^c functions primarily as a copper dependent antioxidant protein.

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4

Prion Protein Peptide

Agents of Death for Neurons

David R. Brown

1. Introduction

The fundamental problem in addressing prion diseases, or the transmissible spongiform encephalopathies, is finding an explanation for the massive neuronal death that occurs. Although some understanding of the mechanism by which neuronal death occurs comes from studies with scrapie-infected mice, most of the insights regarding a possible mechanism have come from cell culture models in which a synthetic peptide (PrP106–126), based on the sequence of the prion protein, has been applied to neuronal cells. This review describes the details of the mechanism of toxicity of this peptide to cells.

2. Prion Diseases

Prion diseases are characterized by neuronal death or neurodegeneration. However, prion diseases are more widely known because of the theory that infectivity is carried by a protein only, namely the abnormal isoform of the prion protein ***(1)***. Despite this controversial and interesting claim, it is probably only relevant to a small proportion of human sufferers of these diseases. The vast majority of cases of human prion disease are Creutzeldt-Jakob disease (CJD), which occurs spontaneously with no known cause ***(2)***. There are also inherited forms of prion disease, which include Gerstman-Sträussler-Scheinker syndrome, fatal familial insomnia, and inherited CJD ***(2,3)***. The number of cases in which transmission of disease has occurred by infection is quite limited. The only confirmed cases are those of iatrogenic transmission

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resulting from transplantation of human tissue, such as dura mater or central nervous system products ***(4)***. The disease, Kuru, is believed to have been spread by eating of human brains. Although there are a number of unequivocal similarities between bovine spongiform encephalopathy (BSE) and the recently described new variant CJD (nvCJD) ***(5,6)***, a causal connection between the two diseases has not been demonstrated.

The human diseases are linked together collectively with several animal diseases, which include BSE of cattle ***(7)***, scrapie of sheep ***(1)***, chronic wasting disease of deer ***(8)*** and transmissible mink encephalopathy ***(9)***, because large amounts of an abnormally folded isoform of the prion protein (PrP^{Sc}) can be detected within the brains of affected individuals ***(2)***. PrP^{Sc} is a derivative of a normal extracellular glycoprotein termed "cellular prion protein" (PrP^{C}). Although widely accepted as being the "prion" or infectious agent of prion disease, PrP^{Sc}, generated by the host, may not be the only component of the infectious agent. Despite this uncertainty, there is little doubt that accumulation of host-generated PrP^{Sc} is the cause of neurodegeneration in these diseases. Nevertheless, the mechanism by which PrP^{Sc} is causative to the neurodegeneration remains unresolved.

PrP^{C} has been shown to be a copper-binding protein ***(10)***, which influences uptake of copper ***(11)***, and also functions as an antioxidant ***(12)*** which protects cells from oxidative stress. Mice deficient in expression of PrP^{C} have reduced activity of the cytoplasmic superoxide dismutase (SOD-1) ***(13)***, probably because of reduced incorporation of copper into SOD-1 ***(14)*** by a number of different cell types. Given that the protein has a rather general function, it is not surprising that it is expressed by a number of different cell types, including neurons ***(15)***, astrocytes ***(16,17)***, microglia ***(18)***, muscle cells ***(19)***, keratinocytes ***(20)***, and various cells of the blood ***(21)***. Nevertheless, the highest level of expression is within the nervous system at endplates and more particularly at synapses in the CNS. Despite PrP^{Sc} accumulation in other tissues in prion disease ***(22)***, it is probably because of this neuronal expression that most PrP^{Sc} accumulates in the central nervous system.

The primary hypothesis concerning the nature of neuronal death in prion disease is that PrP^{Sc} is directly neurotoxic. The chief competing hypothesis is that, in conversion of PrP^{C} to PrP^{Sc}, the normal function of PrP^{C} is lost. This loss of function would then lead to a deterioration in normal cellular metabolism, which would trigger apoptosis in neurons. This theory, as a stand alone description of the cause of neurodegeneration, has been dismissed by studies with mice lacking PrP^{C} expression ***(23)***. Such mice live normally and show no signs of prion disease. It is still possible that another molecule functions in place of PrP^{C}, or that the copper metabolism and/or the oxidative metabolism of PrP^{C}-deficient cells adjusts to deal with the lack of expression. Neverthe-

less, work with cultured neuronal cells indicates that loss of PrP^C function probably is involved in the mechanism of cell death. This mechanism is described in detail below.

3. Neurotoxicity of PrP^{Sc} In Vivo

The principal model of study of prion disease is artificial infection of mice or hamsters with the scrapie agent of sheep. Despite 20 years or more of study of scrapie in rodents, few details have emerged to explain the mechanism behind neurodegeneration in prion disease. Neurons undergo apoptosis at an accelerating rate toward the end of the disease, as symptoms begin to develop before death ***(24,25)***. However, it is not possible to conclude from these studies that cell suicide is responsible for all of the cell death that occurs.

Progression of cell death and its time-course have been studied in detail ***(25–27)***. The occurrence of neuronal loss reflects quite closely the distribution of PrP^{Sc}. However, this distribution and the extent and form of the resulting pathology is variable, and depends on the strain of scrapie agent used to initially infect the rodents ***(28)***. Some aspects have been clearly demonstrated by a number of groups. First, microglia present in the vicinity of PrP^{Sc} become activated before symptoms or any detectable neuronal death ***(24,25,29)***. Second, astrocytosis begins at about the same time that neuronal death begins and is a consistent feature of the disease. Other changes have also been noted such as increases in various cytokines ***(30)***, evidence of oxidative damage, and decrease in activity of nitric oxide synthase ***(31)***. However, the causal connection between any of these changes and neuronal death is unclear.

Furthermore, evidence is missing for PrP^{Sc} being directly toxic in vivo. To date no one has managed to inject PrP^{Sc} or a derivative of the protein into an animal and observed acute neuronal loss. A recent investigation claims that the amount of PrP^{Sc} needed to induce disease in mice by direct intracerebral injection is not sufficient to produce local PrP^{Sc}-specific neurodegeneration ***(32)***. The effect may have been masked by cell death induced by the surgical procedure. However, the implication of this, if true, is that, to be neurotoxic PrP^{Sc} must be generated by the host.

A further observation is that accumulation of large amounts of host-generated PrP^{Sc} is also not sufficient to cause neurodegeneration. PrP^C-knockout mice ***(23)***, that had received grafts of neural tissue overexpressing PrP^C showed accumulation of PrP^{Sc} in PrP^C-deficient tissue (i.e., of their brains), following infection with the scrapie agent, but neurodegeneration was not detectable in the vicinity of this accumulation ***(33)***. This suggests that PrP^C expression by the host is necessary for PrP^{Sc}-induced neurodegeneration.

Although host expression of PrP^C and host generation of PrP^{Sc} appear to be necessary to a toxic model of neurodegeneration, neuronal expression of PrP^C

may not be necessary. Transgenic mice that express PrP^C only in astrocytes have been generated ***(34)***. Such mice do not express PrP^C in neurones, and all PrP^{Sc} generated by such mice upon infection with the scrapie agent must be produced by astrocytes. These can succumb to prion disease and show vast neurodegeneration. This implies that neurodegeneration in prion disease may be caused by PrP^C-expressing astrocytes ***(34)***. However, in these experiments in which there is no neuronal expression of the prion protein, there is also no function of the prion protein in neurons, and the possible causal involvement of lack of neuronal PrP^C function in the neurodegeneration in these mice is unknown.

Recent experimental evidence using transgenic mice expressing a truncated prion protein of 106 amino acids sheds light on what part of the prion protein molecule is necessary for both infection and neurodegeneration ***(35)***. The truncated version of PrP^C expressed by these mice can be converted into a truncated PrP^{Sc} capable of infecting the same transgenic mice, and inducing neurodegeneration. The 106 amino acids comprise the amino residues 89–140 and from 177 to the C-terminus. Other work has implicated the C-terminus of the molecule in conversion of the PrP^C to the abnormal isoform and possible interactions with chaperone molecules, which may aid conversion ***(36,37)***. This is the strongest evidence so far that the region of the protein around amino residues 89–140 is necessary for the toxicity of PrP^{Sc}.

4. Neurotoxicity In Vitro

Further elucidation of the requirements and mechanism of toxicity of host-generated PrP^{Sc} has been found to be difficult in vivo, because of the inability to separate requirements for infectivity and conversion of PrP^C to PrP^{Sc} from those of the resulting neurodegeneration. Most attempts at understanding PrP^{Sc}-induced neurodegeneration have been based on cell culture studies, which have demonstrated that PrP^{Sc} application to neuronal cells results in a toxic effect ***(24,38)***. Use of the full length molecule is difficult, because no true pure source of PrP^{Sc} exists. For such studies PrP^{Sc} is derived from extracts of brains from mice treated with proteinase K. Furthermore, no work with recombinant PrP^C has managed to generate PrP^{Sc}. Although there are many claims of in vitro conversion ***(39)***, these studies either require addition of large amounts of PrP^{Sc} or produce protease-resistant protein that does not appear to be infectious, and only generates multimers and not true fibrils ***(40,41)***.

In an attempt to generate pure protein that mimics PrP^{Sc}, researchers have turned to the use of synthetic peptides. Forloni et al. ***(42)*** were the first to examine the neurotoxicity of PrP to try to identify the toxic domain (amino residues 89–145). They synthesized distinct peptides, based on portions of the human prion protein sequence and demonstrated that one of these, PrP106–126, was fibrillogenic, protease-resistant, and induced neuronal death by apoptosis in

Table 1
Summary of Peptide Qualities

Peptide	Fibril formation	Toxicity	Requirements	
			Microglia	PrP^C
PrP59–66	No	–	na	na
PrP59–91	No	–	na	na
PrP89–106	No	–	na	na
PrP106–126	Yes	++	Yes	Yes
PrP112–126	Yes	+++	Yes	Yes
PrP113–134	Yes	++	Yes	Yes
PrP113–116 (AGAA)	No	–	na	na
PrP113–120 (AGAAAAGA)	Yes	–	na	na
PrP121–134	No	–	na	na
PrP127–147	Minor	–/+	No	No
PrP^{Sc}	Yes	++++	Yes	Yes

Shown are all peptides that have been tested for toxicity. This table summarizes results from various sources ***(24,42,43,49,50,54,71)***. The results for the different peptides are compared to those known for PrP^{Sc}. PrPxxx–xxx indicates the amino residues of the human PrP^C sequence on which the synthetic peptide was based. Under "Requirements," PrP^C indicates need for neuronal PrP^C expression for toxicity; "Microglia indicates" that reduction in microglia content abolishes toxicity. na = not applicable. Number of + under toxicity indicates magnitude of toxicity.

cultured hippocampal cells. The parallels between this peptide's structure and that of PrP^{Sc} mean that it is reliable as a synthetic model of the whole protein, and has been extensively used as such by a large number of groups.

Recently, a reevaluation of the toxicity of PrP106–126 has been carried out ***(43)***. Other peptides, generated around the region of 106–147, were tested to determine the smallest derivative of the human prion protein that mimics the toxic mechanism of PrP^{Sc} with maximal toxicity. A summary of the toxicities of various PrP peptides is shown in **Table 1**. Some peptides, such as PrP127–147 or PrP121–134, show little or no toxicity. The peptide PrP113–120, was also found to be not toxic, however, this peptide was found to form fibrils (**Fig. 1**) and is currently the shortest known fibrillogenic peptide with the palindromic sequence, AGAAAAGA. Additionally, although this peptide was not toxic on its own, it was necessary for the toxicity of larger peptides, such as PrP106–126. The most toxic peptide sequence tested was that of PrP112–126, which peptide corresponds to a region of the protein conserved entirely in every known species in which the prion protein has been sequenced ***(44,45)***. The findings indicate the importance of the AGAAAAGA region of the prion protein. A peptide

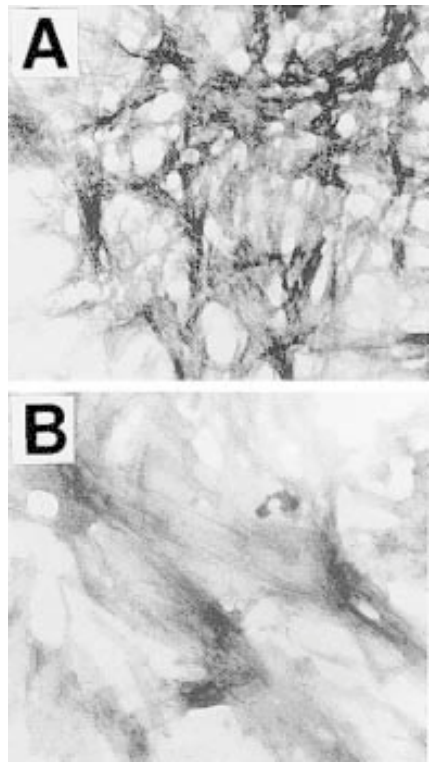

Fig. 1. Fibrils of PrP. Electron microscopic photomicrographs of peptides prepared in phosphate-buffered saline and stained with PTA to produce shadowing. **(A)** PrP106–126. **(B)** AGAAAAGA. These findings make the peptide AGAAAAGA the shortest known fibrillar peptide. Magnification was ×50,000.

based on this region of the protein can also be used to inhibit toxicity of PrP106-126 ***(43)***. Furthermore, deletion of this region of PrP^{C} in transfected cells prevents the formation of PrP^{Sc} ***(46)***. Peptides generated which include the AGAAAAGA region, inhibit in vitro conversion of PrP^{C} to PrP^{Sc}, suggesting that these amino residues are essential for the interaction of the two molecules necessary for the conversion event ***(47)***. From these results, it is likely that this region of the protein will emerge as being the essential component of PrP's protein sequence, regarding both conversion to the abnormal isoform and neurotoxic nature of PrP^{Sc}.

PrP106–126 causes apoptosis of neurons in cultures derived from mouse brain ***(42)***. Before describing what is known about the mechanism of this effect, it is perhaps necessary to point out what kinds of cells are susceptible to this toxicity. However, it is also necessary to point out that the toxicity of PrP106–126 to any

cell appears to be dependent on the presence of an additional factor in the form of some kind of stress such as oxidative stress. PrP106-126 is not toxic to pure populations of neuronal cells. Neuron-like cells of various cell lines, such as neuroblastomas (N2A) or PC12 cells ***(48)***, are not susceptible to the toxicity of PrP106–126 on their own. Several tumors of neuroectodermal origin have been studied, in addition, but these cells are only susceptible to PrP106–126 toxicity if they possess a mixed phenotype (D. R. Brown, unpublished observations). The presence of a source of oxidative stress, such as can be produced by activated microglia, seems to be a critical factor (but not sufficient) for PrP106–126 toxicity.

Under appropriate conditions PrP106–126 is toxic to neurons from the hippocampus, cerebellum and neocortex ***(42,49,50)***. The peptide is also toxic to myotubes ***(19)***, nerve growth factor-differentiated PC12 cells or PC12 cells expressing high levels of PrP^C ***(48)***. In the presence of an inhibitor of cell division, PrP106–126 is also toxic to other cells, such as astrocytes ***(51)*** and microglia ***(52)***. Susceptibility to PrP106–126 toxicity appears to depend on two conditions: only cells expressing relatively high levels of PrP^C are susceptible, and, of these, only, or mostly, nondividing cells are susceptible.

Mice deficient in PrP^C expression cannot be infected with PrP^{Sc} ***(53)***. As indicated above, accumulation of PrP^{Sc} in PrP^C-deficient tissue does not result in toxicity to neurones ***(33)***. However, direct injection of PrP^{Sc} into the brain of PrP^C-expressing (wild-type) mice also does not induce neurodegeneration ***(32)***. Analysis of cerebellar cells and cortical cells from these mice also suggests that the PrP106-126 peptide was not toxic to neuronal cells lacking PrP^C-expression ***(49,54)***. This effect does not result from failure of PrP106–126 to enter neurons. Studies with biotinylated peptide have shown that PrP106–126 enters the same neuronal compartments in PrP^C-deficient neurons as PrP^C-expressing neurons ***(55)***. The lack of toxicity to PrP^C-deficient cells is not caused by greater resistance of these cells to toxic substances. In fact, PrP^C-deficient cells are more susceptible to apoptosis-inducing substances, and to oxidative stress, than are PrP^C-expressing neurons ***(13,54)***. The reason why PrP106–126 (or PrP^{Sc}) addition to PrP^C-deficient cells does lead to increased cell death is presently unknown. However, it is possible that binding of the peptide, either to a receptor ***(56)***, upregulated with PrP^C expression, or to PrP^C itself, may be necessary for activating the appropriate signal transduction cascade within neurons.

Although there is some evidence that toxicity of PrP106–126 increases with increased levels of PrP^C expression ***(57)***, the relationship is not that clear. Studies were also carried out with mice overexpressing PrP^C. However, PrP106-126 was only more toxic to cerebellar cells from one strain of overexpressing mice (Tg35) than to wild-type cells but was not more toxic to cells from another strain (Tg20) ***(58)***. The increased toxicity to the Tg35-overexpressing cerebel-

lar cells was related to changes in the behavior of microglia in the cultures, and not in the metabolism of the neurons. Therefore, above a certain critical level of expression, increasing PrP^C at even higher levels may not increase susceptibility to toxicity.

5. A Role for Microglia

Evidence for a role for microglia in the toxicity of PrP106–126 to neuronal cultures came from two kinds of experiments. Cultures of wild-type (PrP^C-expressing) cerebellar cells were treated with L-leucine methyl ester, a compound that is selectively toxic to microglia. This treatment abolished toxicity of the peptide ***(54)***. At about 4 d in culture (and 4 d of peptide treatment), cerebellar cell cultures consist of approx 85% neuronal cells, 10% astrocytes and 5% microglia. The L-leucine methyl ester treatment reduces microglia content by 80% (D. R. Brown, unpublished observations). Second cerebellar cell cultures treated to remove microglia were co-cultured with increasing numbers of microglia. Increasing microglia content increased the toxicity of PrP106–126 to cerebellar cell cultures ***(54)***.

Such observations on their own, would suggest that PrP106–126 toxicity is indirect and a result of toxic factors released by activated microglia. However, co-culture of wild-type microglia with PrP^C-deficient cerebellar cells did not result in PrP106–126 toxicity to PrP^C-deficient cerebellar neurons ***(54)***. PrP^C expression remained the principal necessity for PrP106-126 toxicity. Nevertheless, microglia in these cultures were necessary for the visible toxic effect.

The toxic agent released by the microglia was found to be superoxide (or reaction products) ***(54)***. Other possible toxic agents released by microglia, such as nitric oxide or tumor necrosis factor were found not to be involved (D. R. Brown, unpublished observations). The necessary quality of microglia in the toxic mechanism of PrP106–126 could be replaced by an alternative source of superoxide, such as xanthine oxidase, an enzyme that generates superoxide. Microglial-reduced cultures of cerebellar cells, incubated with PrP106–126 and xanthine oxidase, lead to PrP106–126 toxicity to the cerebellar cells ***(54)***. Thus, for PrP106–126 to be toxic to PrP^C-expressing neurons, the neurons must be concomitantly exposed to a source of oxidative stress. In support of this it has been found that inhibitors of oxidative stress or antioxidants can inhibit the toxicity of PrP106–126 ***(54)***.

The effect of PrP106–126 on microglia has also been examined. The peptide induces proliferation of microglia, as well as activation ***(52,59)***. Although activated microglia are inhibited from proliferating, it is probable that two subpopulations respond differently to the peptide. Activation of the microglia is accompanied by release of calcium from intracellular stores ***(52)***, and possibly uptake through L-type channels ***(60)***. However, the latter may be related to

death commitment, because PrP106–126 was found to be toxic to a small subpopulation of microglia, when proliferation was inhibited with cytosine arabinoside ***(52)***. PrP106–126 also induces release of the cytokines, interleukin-6 (IL-6) and IL-1 (***61***), from microglia which are probably related to induction of astrocyte proliferation ***(51)***. However, there has been no further reliable analysis of the mechanism by which PrP106–126 activates microglia.

Microglia also express PrP^C ***(18)***. The level of microglia expression alters the ability of astrocytes to be activated by substances such as endotoxins ***(18)***. Cultures from one particular strain of PrP^C-overexpressing mice (Tg35) could be easily activated by lipopolysaccahride whereas activation of microglia from PrP^C-deficient mice resulted in little superoxide release. The reasons for this remain unclear. Analysis of PrP^C-expression in the overexpressing microglia revealed a difference in the level and glycoforms of PrP^C expressed ***(58)***. As well as being more easily activated, the microglia in Tg35 mice proliferated more rapidly both in culture and in vivo. Staining of microglia in sections of cerebellum from wild type and PrP^C overexpressing mice showed higher levels of Mac-1 positive microglia (**Fig. 2**). These Tg35 mice show paralysis and neurological changes as they age, which may be related to higher numbers of activated microglia in their brains.

The relevance of microglial activation to prion disease was confirmed by studies of scrapie-infected mice. Large numbers of activated microglia have been detected in the brains of scrapie infected mice long before any clinical changes can be detected ***(29)***. These changes in microglia occur concurrent with increased accumulation of PrP^{Sc} ***(24)***. However, the changes that occur as the disease progresses are complex and assigning a causal role to microglia from such data would be presumptive.

6. A Role for Astrocytes

Apart from neurodegeneration, the other major change that occurs in prion disease is gliosis, and especially astrogliosis. Increased glial fibrillary acidic protein (GFAP) has been used as a marker for astrogliosis in prion disease. Levels of GFAP increase continuously in all brain regions after PrP^{Sc} is detected ***(62)***. The astrogliosis is typical of the end stage of the disease, and is closely associated with the increased neurodegeneration observed. However, although an environment containing activated astrocytes can be deleterious to neuronal survival, it is unclear whether astrogliosis stimulates neurodegeneration in prion disease.

As with neurotoxicity, application of PrP106–126 to astrocytes in culture has been used to model astrogliosis in vitro. PrP106–126 stimulates astrocyte proliferation and microglia proliferation in mixed glial cultures ***(59)***. However, PrP106–126 did not stimulate astrocytes to proliferate in the absence of

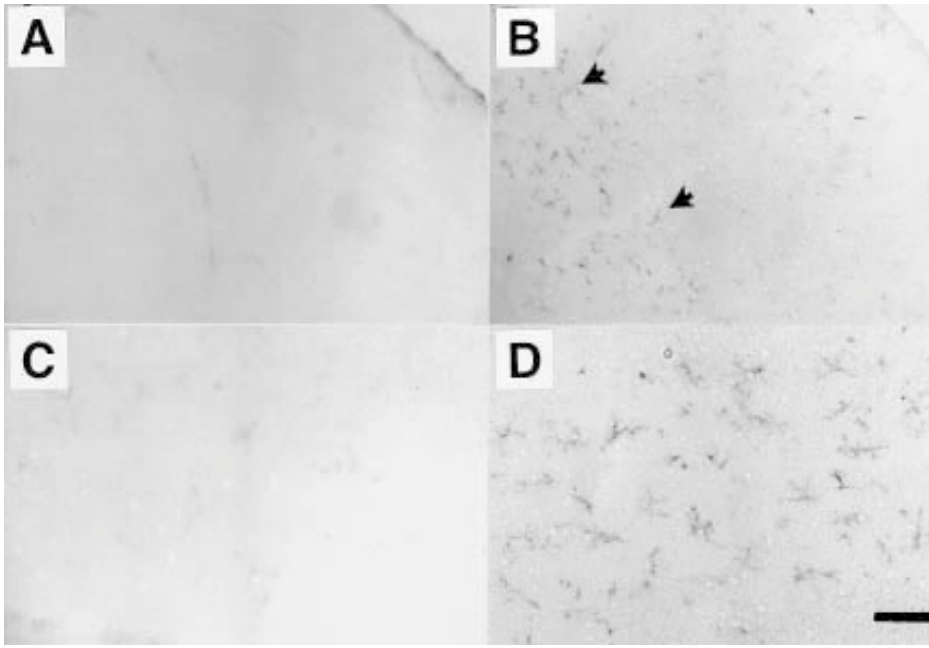

Fig. 2. Microglia. Photomicrographs of sections from adult mouse cerebellum (4–5 mo of age). Wild-type (**A** and **C**) and Tg35 (**B** and **D**) were stained with an antibody to Mac-1. Microglia stained with Mac-1 appear darkly colored (arrowed). No staining was observed in wild-type sections, but large numbers of microglia are present in Tg35 sections. The stained in A is enlarged in C, and that of B is enlarged in D. Scale bar = 400 μ*M* in A and B and 100 μ*M* in C and D.

microglia. Pure cultures of astrocytes did not show increased proliferation when PrP106–126 was added ***(59)***. However, the presence of microglia alone was not sufficient for PrP106–126 to induce astroglial proliferation. Like microglia and neurons, astrocytes express PrP^C in culture ***(17)***. PrP^C-deficient astrocytes did not proliferate in the presence of both PrP106-126 and wild-type astrocytes ***(51)***. Therefore, astrocytic PrP^C -expression is necessary for PrP106–126 induced astrocytic proliferation. Furthermore, conditioned media, from microglia treated with PrP106–126, was insufficient to induce astrocytic proliferation ***(18)***. Similarly, conditioned medium from microglia treated with granulocyte-macrophage-colony-stimulating factor (GM-CSF), a mitogen of microglia, had only a minor effect on astrocytic proliferation, implying that the proliferation of astrocytes induced by PrP106–126 in the presence of microglia, is not simply a result of cytokines released by microglia. However, conditioned medium from GM-CSF-stimulated microglia did greatly enhance the

proliferation of astrocytes in the presence of PrP106–126. This implies that PrP106–126 primes astrocytes to be more sensitive to stimulation from cytokines, such as IL-1 and IL-6, released by microglia. This implies that PrP106-126 also has a direct effect on astrocytes necessary for stimulation of astrocytic proliferation.

PrP106–126 has other direct effects on astrocytes. PrP106–126 has been shown to inhibit the uptake of glutamate ***(63)***, an important neurotransmitter in the central nervous system. Clearance of glutamate after its release at synapses is necessary to protect neurons from the excitotoxic effects of glutamate. Thus, inhibition of glutamate uptake by astrocytes may be another way that PrP106–126 could indirectly cause neurotoxicity.

The role of astrocytes in the neurotoxicity of PrP106–126 to cerebellar cell cultures has been assessed. Treatment of cerebellar cells with α-aminoadipic acid, a substance that selectively kills type 1 astrocytes ***(64)***, does have a minor effect in inhibiting PrP106–126 neurotoxicity ***(17)***, but not enough for this to be a major contributing factor in the toxicity of PrP106-126 to this system. For freshly prepared cerebellar cell cultures on their own, the involvement of astrocytes in PrP106–126 toxicity was dismissed ***(54)***.

Recent studies ***(65,66)*** have advanced understanding of the interaction of neurons with large numbers of astrocytes which is more typical of later stages of scrapie, following gliosis. In the presence of large numbers of astrocytes, cerebellar neurons become dependent on astrocytes for protection from glutamate toxicity. Coculture of neurons with astrocytes increases the velocity of uptake of glutamate by astrocytes. The coupling of astrocytes and neurons in culture, in this manner results in an increased susceptibility of neurons to the toxicity of glutamate, but, in the presence of astrocytes actively clearing glutamate this has little consequence. This increased sensitivity to glutamate of neurons may represent an enhanced efficacy of glutamatergic synapses. However, an in vivo correlate of this is still unknown. The implication for glutamate toxicity is that any substance that activates astrocytes, or inhibits their ability to clear glutamate, will expose neurons to the toxicity of glutamate.

PrP106–126 is not toxic to neurons deficient in PrP^C ***(49)*** and PrP106–126 only inhibits glutamate uptake by PrP^C-expressing astrocytes ***(63)***. A coculture system between PrP^C-deficient neurons and PrP^C-expressing astrocytes was prepared. In such a system micromolar concentrations of glutamate were found not to be toxic to PrP^C-deficient neurons, although inhibition of astrocytic glutamate clearance by PrP106–126 caused neuronal loss because of glutamate toxicity ***(17)***. However, the concentration of glutamate present in the culture during such experiments was not sufficient to kill PrP^C-deficient neurons in the presence of PrP106–126 without astrocytic co-culture. In this system, the dependence of PrP^C-deficient neurons on astrocytes, for protection from

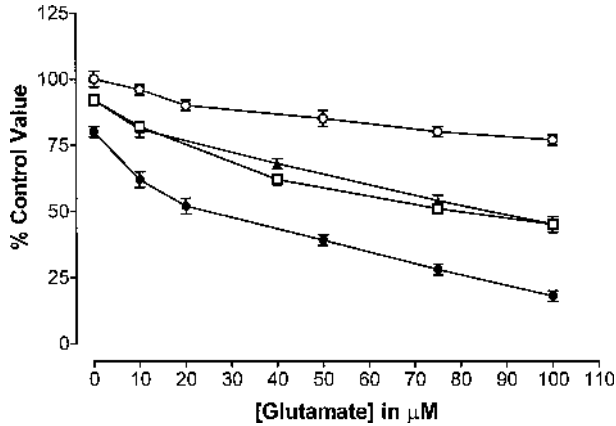

Fig. 3. PrP106–126 is not toxic to PrP-deficient cerebellar cells. PrP-deficient cerebellar cells were cultured alone or co-cultured with wild-type (PrP-expressing) astrocytes. The cells were exposed to increasing concentrations of glutamate with or without addition of 80 μ*M* PrP106–126. After 4 d of treatment, the survival of the PrP-deficient cerebellar cells was determined by carrying out an MTT assay on the cerebellar cells only. Co-culture with wild-type astrocytes (O) significantly (Student's *t*-test, $p < 0.05$) reduced glutamate toxicity, compared to the toxicity of glutamate to cerebellar cells not co-cultured (□).PrP106–126 did not enhance the toxicity of glutamate to cerebellar cells not co-cultured (▲). However, PrP106–126 greatly enhanced the toxicity of glutamate to cerebellar cells co-cultured with astrocytes (●). PrP106–126 enhanced glutamate toxicity at all concentrations and dramatically abolished the protective effects of astrocytes on protection of neurons against glutamate toxicity. The toxic effect of the glutamate in the presence of PrP106–126 and astrocytes was greater than if the astrocytes had not been present.

glutamate toxicity, meant that inhibition of glutamate uptake by PrP106–126 stripped the PrP^C-deficient neurons of their protection, and resulted in a glutamate induced toxic effect. Thus, in this system, PrP106–126 can have an indirect toxic effect on neurons by interfering with neuron-astrocyte interactions (**Fig. 3**).

It is possible that these results only represent a curious cell culture effect. However, there are reasons to suggest that this is not the case. Mice have been generated that expressed PrP^C via a GFAP promoter (Tg3/$Prnp^{o/o}$ mice) ***(34)***. In these mice, only astrocytes (and not neurons) express PrP^C. After infection with the scrapie agent, these mice developed neurodegeneration and accumulation of PrP^{Sc} similar to prion disease. It is possible the neurodegeneration seen following scrapie infection of these mice, which is similar to that seen in prion disease, comes about either wholly or in part as the result of glutamate

toxicity. Indeed, experiments using the mice described in Raeber et al. ***(34)*** supports this ***(17)***. PrP106–126 was only toxic to these cultures when excess Tg3/$Prnp^{o/o}$ astrocytes (which are $Prnp^{+/+}$) were added to cerebellar cell cultures from Tg3/$Prnp^{o/o}$ mice. It is likely that PrP106–126 was not toxic to cerebellar cell cultures without co-culture, because there were insufficient numbers of astrocytes present. This confirms that PrP106–126 can only be toxic to PrP^{C}-deficient neurons in the presence of excess numbers of PrP^{C}-expressing astrocytes. Previous work on the phenotype of PrP^{C}-deficient neurons suggests that they are compromised in their ability to resist oxidative stress ***(13)***. PrP106–126 is known to have effects on PrP^{C}-deficient neurons that also alter their resistance to oxidative stress ***(13,54)***. The neurons in the mice of Raeber et al. ***(34)*** are PrP^{C}-deficient. As PrP106–126 alters the wild-type neuronal phenotype to one similar to that of a PrP^{C}-deficient neuron then possibly there is no contradiction between their findings and that of Brandner et al. ***(33)***, who suggest that PrP^{C} expression is necessary for the neurodegeneration seen in prion disease. By the late stages of prion disease neurons may be devoid of functional PrP^{C} because of conversion to PrP^{Sc}, and may be phenotypically PrP^{C}-deficient.

Collectively, these observations imply a complex picture of the toxicity of PrP106–126 or PrP^{Sc}. However, they also emphasize the likely role played by glia in toxicity. Microglia may play a role in initiating indirect aspects of neurotoxicity, and also in inducing astrocyte proliferation. Neurodegeneration may also be accelerated by astrogliosis, and complex interactions between neurons and astrocytes may imply that the massive round of apoptosis occurring at the late stages in the disease are a result of glutamate toxicity. Inhibiting the response of *N*-methyl-D-aspartate receptors may thus prove to be beneficial in slowing disease progress.

7. Loss of Function

Despite the clear role of glia in cell culture models of PrP106–126 toxicity, the foregoing discussion indicates that glial effects, although necessary, are not sufficient for induction of neuronal loss. The foremost indication for this is the necessity for PrP^{C} expression. Neither PrP106-126 nor PrP^{Sc} was toxic to cultures of cerebellar cells from PrP^{C}-deficient mice ***(24,49)***. Combining PrP^{C}-deficient neurons with PrP^{C}-expressing microglia did not alter the result: PrP106–126 was not toxic to these cells in this situation. As described above, combining PrP^{C}-deficient neurons with PrP^{C}-expressing astrocytes did create a system in which PrP106–126 was toxic, but this effect was dependent on the presence of glutamate. Furthermore, this latter experiment does not explain how PrP106–126 is toxic to wild-type cerebellar cell cultures.

Treatment of wild-type neurons or PC12 cells with PrP106–126 leads to a drop in cellular resistance to oxidative stress *(13,48,54)*. This implies that cerebellar cells are more prone to the toxicity of oxidants, such as superoxide. PrP^C-deficient neurons do not show an increased sensitivity to oxidants in the presence of PrP106–126. Despite this, there is evidence that PrP106–126 alters the metabolism of PrP^C-deficient cells *(67)*. However, phenotypically, neurons and astrocytes *(13,51)* from PrP^C-deficient mice are more susceptible to oxidative stress, compared to wild-type cells. Spontaneous apoptosis of granular cells in cerebellar cell cultures occurs at a higher rate within the first 2 d of culture than in wild-type cultures. This difference can be blocked with antioxidants applied to the culture *(48)*, or by transfection of the cells with either PrP^C or Bcl-2 *(68)*. Increased expression of PrP^C in PC12 cells is linked to an increase resistance to oxidative stress and copper toxicity *(48)*.

The effect of PrP106–126 on the activity of various antioxidant proteins was also examined to determine why neurons become more sensitive to oxidative stress when treated with PrP106–126. Cerebellar cells treated with PrP106–126 showed decreased activity of the enzyme Cu/Zn SOD1 *(48,69)*, a cytoplasmic protein involved in breaking down superoxide (**Fig. 4**). Other enzymes, such as glutathione peroxidase and catalase, showed no sign of change in activity.

Once again, there is a parallel between cells treated with PrP106-126 and cells from mice deficient in PrP^C expression and PrP^C activity. Extracts from cerebellar cells and brains of PrP^C-deficient mice also had lower levels of SOD activity *(48)*. Further analysis of the phenotype of PrP^C-deficient mice indicated that this reduction in activity was not caused by downregulation of superoxide dismutase expression but was due to decreased incorporation of copper into the molecule *(14)*. Despite this, PrP106–126 also directly inhibited the activity of Cu/Zn superoxide dismutase *(48)*.

The parallels between the phenotype of PrP^C-deficient cells and cells treated with PrP106–126 suggest that PrP106–126 may induce a loss of function. This would suggest that PrP106–126 can directly inhibit the function of PrP^C. Recent work indicates that PrP^C itself is an antioxidant enzyme *(12)*. However, there is no clear evidence yet that PrP106–126 directly inhibits this activity. However, there is sufficient evidence to suggest PrP106–126 impairs neuronal resistance to oxidative stress. Furthermore, studies on scrapie-infected mice have found evidence of oxidative damage and mitochondrial disfunction indicative of oxidative stress *(70)*. These findings give credence to the results from cell culture studies with PrP106–126.

8. Summary

The mechanism of toxicity of PrP106–126 has been examined in detail. Although some gaps in the knowledge of its action are still present, there is

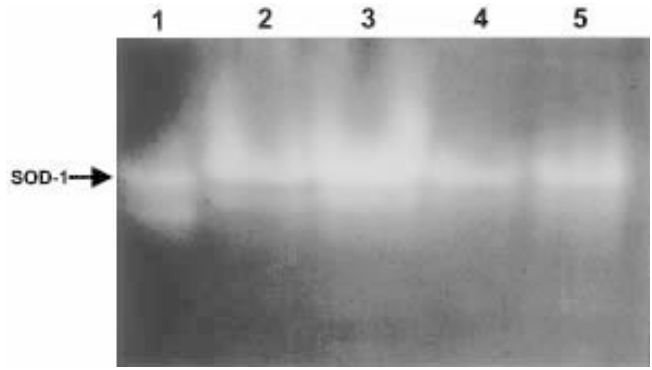

Fig. 4. The activity of Cu/Zn superoxide dimutase (SOD-1), from extracts of cells treated with PrP106–126, was determined. This assay is based on protein electrophoresis in a nondenaturing acrylamide gel. After electrophoresis, the protein bands corresponding to SOD1 can be visualized in the gel, by exposing them to a source of oxygen radicals and nitro blue tetrazolium, which produces a blue color when the radicals are detected. Clear bands represent the activity of SOD. Lane 1, pure bovine SOD; lanes 2–5 are samples from wild-type cerebellar cells treated with either vehicle (2), xanthine oxidase (3), or 80 μ*M* PrP106-126 (4) or 20 μ*M* (PrP106–126) for 1 d. Xanthine oxidase, a source of oxidative stress, causes an increase in the intensity of the bands, corresponding to SOD-1 activity. PrP106–126 causes a reduction in activity.

sufficient evidence to create a theory of its action. This theory can also be assessed by observing what is known about neurodegeneration in models such as mouse scrapie.

A schematic representation of PrP106–126 toxicity is shown in **Figure 5**. PrP106–126 aggregates to form fibrils. This peptide is capable of binding to proteins on the surface of different cell types, and can be internalized ***(55)***. PrP106–125 is toxic to neuronal cultures containing a mixture of other cells, such as microglia and astrocytes. For this toxicity to occur, PrP106–126 must interact with neurons and glia. PrP106–126 activates microglia, causing them to release superoxide and cytokines. The cytokines initiate astrocytic proliferation. PrP106–126 interacts with astrocytes, priming them to respond to mitogens from microglia and inhibiting glutamate uptake. The direct effects of PrP106-126 on neurons blocks the activity of PrP^C, and this directly or indirectly leads to diminished protection of the injured neurons to oxidative stress. In this susceptible state, PrP106–126 treated neurons are assaulted by toxic substances in the environment, such as superoxide and glutamate which initiates signal transduction cascades, leading to increased uptake of calcium ***(50)*** and ending in neuronal apoptosis. Further investigation of animal paradigms, such as mouse scrapie, will determine if this in vitro model is relevant to true prion disease.

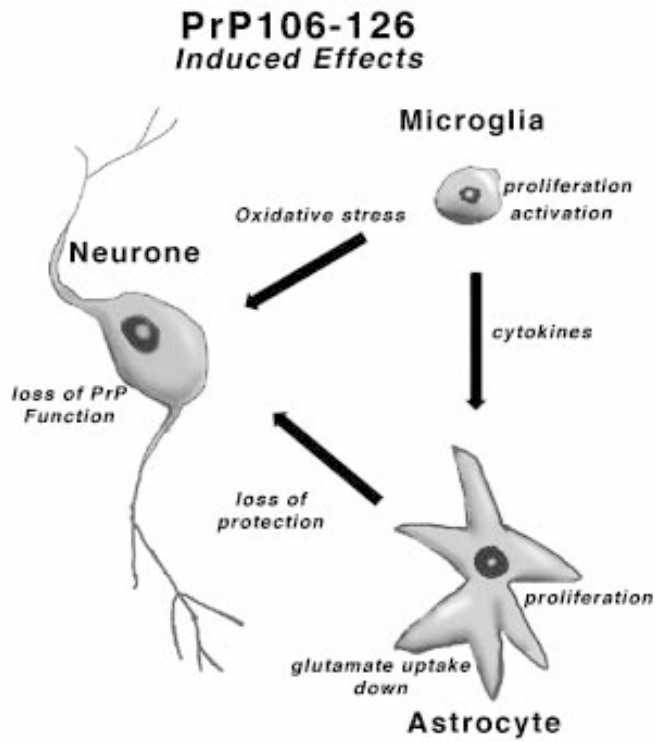

Fig. 5. Summary figure showing the details of the theoretical toxic mechanism of PrP106–126 to neurons, as described in the text.

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5

Characterization of Bovine Spongiform Encephalopathy and Scrapie Strains/Isolates by Immunochemical Analysis of PrP^{Sc}

Martin H. Groschup, Frauke Junghans, Martin Eiden, and Thorsten Kuczius

1. Introduction

In the past two decades, thoroughly standardized mouse incubation time and brain lesion profile scoring assays have been developed to discriminate between prion strains. However, in these mouse infection experiments, large numbers of animals (about 20 mice/line) from three different highly inbred mouse lines (C57Bl, VM95, RIII), plus their intercrosses, need to be infected, and their brain tissues subsequently examined ***(1–8)***. Although results obtained are highly reliable, the effort and time needed for conducting these experiments are considerable. Therefore, alternative criteria and techniques have been developed to characterize transmissible spongiform encephalopathy (TSE) agents. Prion infections are accompanied by the accumulation of an abnormal isoform (designated PrP^{Sc}) of normal host-encoded prion protein (PrP^{C}). Both isoforms have the same amino acid sequence and molecular mass, but differ significantly in their three-dimensional structure and biochemical characteristics. The three-dimensional structure of PrP^{C} is characterized by a high α-helical content ***(9)***; all or part of it undergoes a posttranslational modification to β-sheet in PrP^{Sc} ***(10–12)***. Although PrP^{C} (33–35 kDa) is completely hydrolyzed by protease treatment, PrP^{Sc} is partially resistant to proteinase K(PK) as 62 N-terminal amino acids are cleaved, leaving a core fragment of approx 141 amino acids (27–30 kDa) unhydrolyzed ***(13)***.

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Kascsak et al. described, for the first time, differences in the molecular masses of nonglycosylated PrP^{Sc} fragments of scrapie strains after proteinase K digestion, i.e. PrP^{Sc} of strain 87V had a slightly smaller molecular mass than the PrP^{Sc} of most other scrapie strains studied (about 19 kDa, compared to 21 kDa) ***(14,15).*** A similar difference was later noted for the two known and well-characterized transmissible mink encephalopathy strains, "hyper" and "drowsy" ***(16,17)***.

Collinge et al. ***(18)*** eventually analyzed PK-treated PrP^{Sc} from Creutzfeldt-Jakob disease (CJD) and variant (v)CJD cases, and found three different molecular masses for the nonglycosylated compound: 21, 20, and 19 kDa. PrP^{Sc} from all vCJD cases clustered in the 19-kDa type. However, the nonglycosylated 20 kDa PrP^{Sc} type has not been observed by others ***(19,20)***. This discrepancy may partly result from the presence or absence of ethylendiamine tetraacetic acid (EDTA) during the PK treatment. Wadsworth et al. ***(21)*** could show that the addition of this metal ion binding compound leads to a smaller cleavage product of 19 kDa (designated type 1* or 2*, respectively), instead of PrP^{Sc} types 1 (21 kDa) and 2 (20 kDa).

Apart from the different molecular masses of nonglycosylated PrP^{Sc}, Collinge et al. also noted differences in the ratios of the three glycoforms of PrP^{Sc} ***(18)***. PrP^{C} precursor protein is glycosylated twice at asparagines at positions 182 and 198 of the protein ***(22)***. As a result of this process non-, mono-, and diglycosylated PrP^{Sc} fractions are accumulated in infected cells. However, although monoglycosylated PrP^{Sc} predominates in all conventional human TSE cases analyzed to date, vCJD PrP^{Sc} is characterized by a predominance of diglycosylated PrP^{Sc} ***(18)***.

By combining the two results, molecular mass determination and glycotyping, it became clear that vCJD represented a novel type of prion disease in humans. Because PrP^{Sc} derived from bovine spongiform encephalopathy (BSE)-infected mice had shown similar molecular characteristics, such as low molecular mass and heavily stained diglycosylated PrP^{Sc}, a link between both diseases has been proposed.

We have recently found that PrP^{Sc} from different experimental and field BSE, and scrapie strains/isolates, are characterized by differences in their resistance to long-term exposure to proteinase K. Hence, the determination of the long-term proteinase K (PK) resistance can reveal additional information on the underlying prion strain.

2. PrP^{Sc} Glycotyping, Molecular Mass Determination, and Long-Term Proteinase K Resistance Analysis: Basic Protocol

The determination of molecular masses and relative proportions of non-, mono-, and diglycosylated PrP^{Sc} are frequently summarized under the term

"glycotyping," although both relate to different molecular characteristics. Therefore, in this chapter, both designations are used in their strict sense, and "glycotyping" refers to the determination of the glycoform ratios only.

2.1. Sample Preparation

PrP^{Sc} containing samples are homogenized. Tissues are cut into small pieces and put into glass homogenizers of appropriate size or syringes with needles of subsequently lower diameters. A number of different lysis buffers have been described in the literature ***(18–20,23)***. Good results are obtained using phosphate buffered solution supplemented with 0.5% NP-40 and 0.5% sodium desoxycholate, which is added to yield a 10% (wv) homogenate. Cells are thoroughly disintegrated by 20–30 plunger strokes, and residual tissue clumps further broken by 30–60 s high-power sonification in a closed vessel (for safety reasons). Coarse debris is then removed by centrifugation for 5 min at 325*g*, and aliquots stored in the freezer at –20°C until further use. PrP^{Sc} signals in homogenates, which contain detergents (particularly, sarkosyl or sodium dodecyl sulphate [SDS]), can fade away within weeks, even if homogenates are kept at –20°C, and are not thawed intermediately. This deterioration seems to affect all three PrP bands to the same degree. Therefore, tissue homogenates should be rapidly processed and analyzed. Sonification of repeatedly thawed samples may partially restore the signals. Samples are eventually digested by PK at conditions chosen according to particular needs (*see* **Subheading 3.2.**). The digestion is terminated by addition of 5 m*M* phenylmethylsulfonylfluoride (obtainable from Boehringer, Mannheim, Germany) and/or heat (70°C, 10 min).

Alternatively, scrapie-associated fibrils (SAFs) can be used. SAFs are purified according to previously published protocols ***(24)*** which also include a PK digestion step (10 μg/mL, 1 h, 37°C). PrP^{Sc} signals in SAF preparations also tend to fade away over time.

The choice of sample preparation depends mostly on the expected PrP^{Sc} content in these tissues. High PrP^{Sc} concentrations are found in brain tissues from patients with CJD, vCJD, kuru, and Gerstmann-Sträussler Scheincker disease, so that homogenates can readily be used. The same applies for brain tissues of scrapie-infected sheep, hamsters, and mice. In contrast, tissues from patients with familial fatal insomnia and cattle with BSE contain much lower amounts of PrP^{Sc}, so that a previous concentration step may be necessary. This is also recommended when brain areas with low PrP^{Sc} concentrations are used.

2.2. Gel Electrophoresis and Immunoblot

Samples are subsequently boiled in sample buffer (50 mM Tris, 117 mM Saccharose, 2% [w/v] SDS, 1% [w/v] β-mercaptoethanol, 0.01 % (w/v) bro-

mphenol blue, pH 6.8) for 5 min. It should be noted that SDS concentrations used in sample buffers vary considerable (0.5–5%) between laboratories. Moreover, different concentrations of reducing substances, such as dithiothreitol or mercaptoethanol are used, with possible impact on the protein separation during electrophoresis.

Samples are run on 12–16% SDS-polyacrylamide gel electrophoresis (PAGE) gels (0.75 mm thickness) at about 10 V/cm, using a commercial minigel apparatus, and proteins are electrotransferred (5 mA/cm^2 for 50 min) onto PVDF (available from Immobilon-P, Millipore, Bedford, MA) membranes, using a commercially available semidry blotting apparatus. It may be necessary to check an even protein transfer over the whole electrode area by blotting standard protein mixtures.

Membranes are then blocked by incubation in 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween-20 (phosphate-buffered saline [PBS-]Tween). Sheets are incubated for 2 h with antibodies in PBS–Tween supplemented with 5% nonfat dry milk. After three washes in PBS–Tween, the sheets are incubated for another 90 min with horseradish peroxidase- or alkaline phosphatase-conjugated antibodies in PBS-Tween. The addition of 5–10% fetal calf serum to the blocking and/or the antibody solution may reduce nonspecific background reactions.

Horseradish peroxidase- or alkaline phosphatase-conjugated antirabbit IgG or conjugated antimouse immunoglobulin IgG are used as detection antibodies. After another three washes, antibody binding is visualized by chemiluminescence substrates, which emit light upon reduction by the enzyme. In earlier studies, we have employed the ECL system (Amersham), but obtained comparable results using other systems. The CDP-Star system (available from Tropix, Bedford, MA) has the advantage that the light emission lasts for a long time which improves the recording of gels by CCD-camera or chemiluminescent phosphor screen. Only clear and discrete banding signals of PrPSc should be recorded. Air bubbles during electrotransfer of the antigens result in uneven signals (holes) and render the results uninterpretable.

3. GLYCOTYPING

3.1. Choice of Samples.

Homogenates, as well as SAF preparations, may be used for glycotyping studies, because comparable results were obtained using sheep scrapie samples (M. Groschup and J. Madec, personal observations). However, the choice of tissue samples may have an impact on the glycotyping results, although controversial results are reported in literature: while Somerville ***(25)*** saw differences in glycotypes in murine PrP^{Sc} derived from different brain areas, such

effects were not observed by others ***(26–28)***. Substantial source-dependent effects may be seen, however, when PrP^{Sc} from peripheral tissues, such as spleen, tonsil, and uterus, are studied ***(29)***. It is therefore recommended that only PrP^{Sc} from similar tissue samples be compared.

3.2. PK Treatment

A number of different PK concentrations have been used, according to the literature, ranging between 10 and 100 μg/mL (final concentration). In the Tuebingen laboratory, PK concentrations of 50 μg/mL are generally used. PK is a rigid enzyme that survives repeated freezing thawing cycles, or prolonged storage at room temperature, without substantial loss of activity ***(30)***.

Samples are usually digested for 1 h at 37°C, although longer times or higher temperatures have also been reported. It is important to note that complete band shift in immunoblots of diglycosylated PrP^{Sc} to a band of molecular mass about 6–7 kDa lower should be achieved and monitored to ensure complete PK digestion. Incomplete cleavage and residual PrP^{Sc} shows as an extra band (*cap*) on top of the immunoblot signal of digested PrP^{Sc}. If there is residual uncleaved PrP^{Sc}, the cleavage conditions must be improved, to ensure complete cleavage, since a mixture of cleaved and uncleaved glycoforms in the stained PrP^{Sc} bands renders any glycotyping analysis impossible.

3.3. Immunoblotting

A number of different antibodies diluted in PBS-Tween at appropriate concentrations, can be used for the immunoblot detection of PrP^{Sc}. For labeling murine PrP^{Sc} we use a polyclonal anti-peptide antibody raised to the N- terminus of mouse PrP (amino acids 95 –110: THNQWNKPSKPKTNMK) ***(31)***. To detect hamster or human PrP^{Sc}, the monoclonal antibody 3F4, is used ***(32)***. Ovine and bovine PrP^{Sc}s are immunostained by using the monoclonal antibodies mab P4 (amino acids 89–104: GGGGWGQGGSHSQWNK) or L42 (amino acids 145–163: GNDYEDRYYRENMYRYPNQ) ***(31,33)***. Mab L42 also reacts with human PrP^{Sc}. Apart from these antibodies, a large range of other mono- and polyclonal antibodies have been used in the literature ***(15,18–20,23)***. However, the choice of antibody can have a remarkable impact on the results obtained, thereby making comparisons of results between laboratories more difficult. As shown on **Figure 1** mAb P4 yields profiles for PrP^{Sc} from ovine cases that are distinguishable from those of bovine cases; use of mAb L42 does not allow such a distinction.

3.4. Recording and Evaluation of Immunoblot Results

PrP^{Sc} protein patterns usually show the three typical PrP bands representing di-, mono-, and nonglycosylated PrP. Initial analysis relied on naked eye

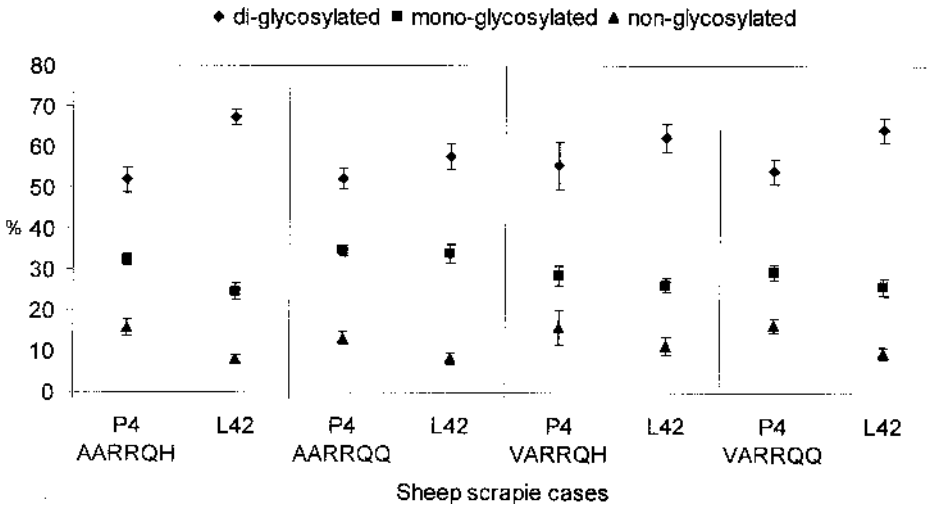

Fig. 1. Proportions of PrP^{Sc} glycoforms in four different sheep genotypes after PK treatment. PrP^{Sc} was purified from sheep cerebellum homogenates. Fibrils were subsequently exposed to proteinase K (50 μg/mL) for 1 h at 37°C. Proteins were separated by 16% SDS-PAGE gels. PrP^{Sc} bands were revealed by immunoblotting using the monoclonal antibodies, mAb P4 and L42, respectively. mAb P4 is directed to amino acid sequences 89–104 and mAb L42 to residues 145–163 of ovine PrP. Horseradish peroxidase-conjugated affinity-purified goat antimouse IgG served as detection antibody. Membranes were developed using a chemiluminescence enhancement kit, and signal scanned electronically. To glycotype PrP^{Sc} from isolates, banding intensities of the di-, mono- and non-glycosylated isoforms were determined and calculated as percentages of the total signal. The percentages of the di-, mono-, and nonglycosylated isoforms are represented as means of at least seven separate gel runs ± SEMs.

examination of blackened autoradiography films, which were exposed to light-emitting immunoblots. However, the limiting blackening capacity of such films may significantly distort the profiles obtained. Therefore, visual evaluation can easily lead to wrong interpretations, if samples are not applied in suitable and comparable amounts. This may also apply for profiles obtained by densitometer scanning of films. The suitable range of the detection and visualization systems used can be determined by comparing signal intensities of serially diluted samples. The discrimination power of electronic imaging devices is substantially greater than the ability of the naked eye for detecting strain-specific features. Electronic scanning of glycoprotein patterns should therefore be the technique of choice, because specific patterns can be distinguished over a much wider range.

4. Molecular Mass Determination

4.1. Choice of Tissue Source

As is the case for glycotyping, estimates of the molecular mass determined for PrP^{Sc} may vary, depending on the chosen tissue. Accordingly, only PrP^{Sc} coming from the same tissue source should be compared.

4.2. Enzyme Treatment

It is generally sufficient to digest PrP^{Sc}-containing samples such as brain homogenates from humans or sheep with PK (50–100 μg/mL) for 1 h at 37°C. In some cases, an extension of this digestion time may be useful, in order to obtain a clear-cut band for nonglycosylated PrP^{Sc}. It is important to avoid EDTA and other ion-binding compounds during the sample preparation steps because these may affect PK cleavage sites, and lead to altered molecular masses of PK treated PrP^{Sc}.

In cases in which nonglycosylated PrP^{Sc} compounds are only scarcely detected by antibodies, proteins may be deglycosylated prior to immunoblot analysis. For this purpose samples are digested with 50 U/mL PNGaseF (available from Boehringer) for 6 h at 37°C. The reaction is terminated by heating to 95°C for 15 min, and samples are stored at –20°C.

4.3. Gel Electrophoresis and Immunoblotting

To obtain a better separation, e.g., for molecular mass determinations, long maxigels of similar thickness may be used. The buffer system can also have an impact on the separation. Tris/Tricine gels are frequently used for the separation of peptides in the molecular mass range 0.5–40 kDa. However, in our experiments such gels performed no better than conventional Tris-HCl or Trisborate-buffered systems, for the separation of PrP^{Sc} bands. Moreover, because "smile" effects may happen in outer gel lanes, comparisons of molecular masses of bands should preferably only be made for antigens run on inner lanes of the gels.

In polyacrylamide gels, charged proteins migrate as they are pulled forward by the electric field, and their migratory speed generally correlates with their size (molecular mass). Depending on the extent of unfolding achieved by boiling in SDS-containing sample buffer, differences in their shape in the native state should be negligible. However, proteins may not be charged and unfolded to the same degree by the pretreatment. Moreover, sometimes they carry intrinsic charges because of their glycosylation, phosphorylation, glycosylphosphatidylinositol anchor attachment, or other posttranslational modifications, which possibly affect the SDS-PAGE results.

In order to better control the separation performance of the gel used, and to predict the distance in migration location, the authors have constructed a protein marker ladder in the molecular range 16–21 kDa. For this purpose, six

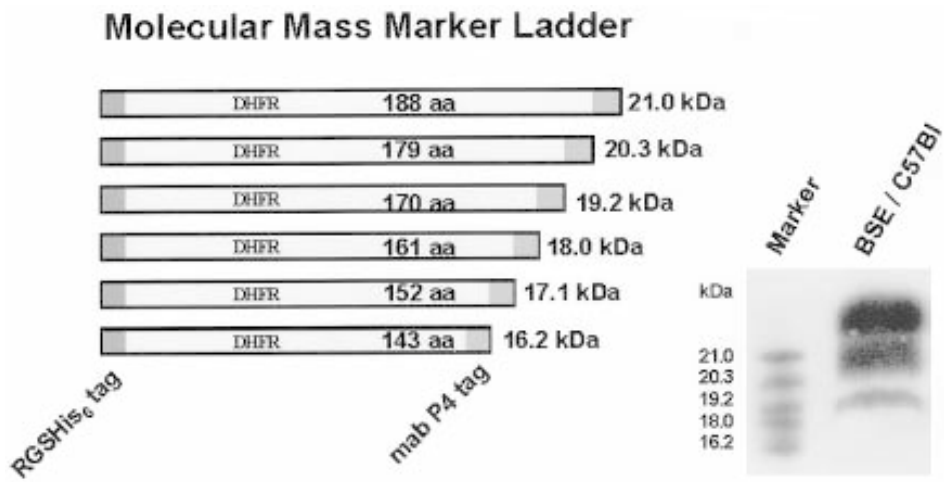

Fig. 2. A novel molecular mass marker ladder covering the range of 16–21 kDa was designed, which can be used in immunoblots for analyzing the size differences of proteinase K treated nonglycosylated PrP^{Sc} fragments. For this purpose, six open reading frames, encoding dihydrofolate reductase fragments in combination with two epitopes of well-characterized monoclonal antibodies (mAb P4 epitope; His-tag epitope), were expressed in *Escherichia coli* and purified markers mixed and separated on gels at appropriate concentrations. Markers are stained by conventional immunoblot detection, using either one of the monoclonal antibodies.

deletion mutants of an open reading frame, encoding deletion mutants of dihydrofolate reductase were fused by a polymerase chain reaction with a sequence encoding the mAb P4 epitope (ovine PrP). Moreover, the open reading frame also encoded a stretch of six histidines. After cloning into a bacterial expression vector, these marker proteins were produced and purified by metal-affinity chromatography. The set of six marker proteins can now be used as an antigen ladder of defined molecular masses: 16.2, 17.1, 18.0, 19.2, 20.3, and 21.0 kDa. Marker antigens are visualized in immunoblots, either by use of mAb P4 or an His-tag specific monoclonal antibody (**Fig. 2**).

4.4. Long-Term PK Resistance Analysis

Using SAF preparation protocols, PrP^{Sc} is purified from brain tissue samples from infected individuals. Alternatively, homogenates may be used. PrP^{Sc} is subsequently exposed for prolonged times (from 1 to 48 h) to PK (50 μg/mL), in order to determine their relative proteolytic stabilities (**Fig. 3**). Nondegraded PrP^{Sc} compounds are visualized by immunoblot. The kind of antiserum used makes no difference. Residual antigen amounts are eventually quantified, using

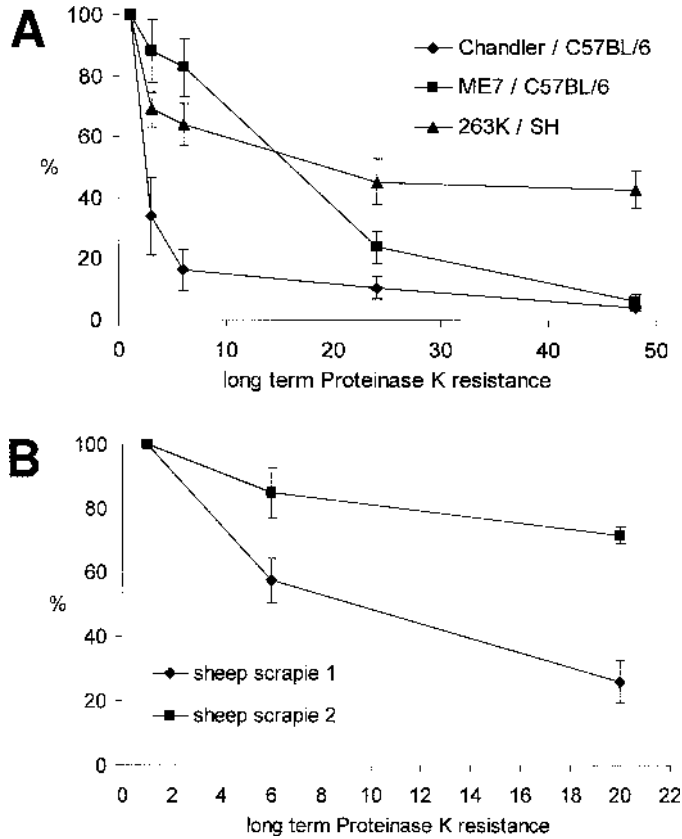

Fig. 3. Analysis of the long-term proteinase K (PK) resistance of mouse and hamster adapted scrapie strains (**top**) and sheep scrapie isolates (**bottom**). Strains ME7 and Chandler were propagated in C57BL/6 mice, and strain 263K in Syrian hamsters. Sheep PrP^{Sc} was isolated from natural infected sheep by scrapie. PrP^{Sc} was purified and fibrils were subsequently exposed for prolonged times (1, 3, 6, 24, 48 h [in the case of the strains]) and for 1, 6, 20 h ([in the case of the sheep isolates]) to PK (50 μg/mL) at 37°C. After SDS-PAGE and immunoblotting protein bands were detected by using the polyclonal antibody Ra5/7 (mice), mAb 3F4 (Syrian hamster), and mAb P4 (sheep). Horseradish peroxidase-conjugated affinity-purified goat antimouse and goat antirabbit immunoglobulin IgG served as detection antibodies. Membranes were developed using a chemiluminescence enhancement kit, and residual PrP^{Sc} compounds recorded electronically. Signal intensities after digestion with PK for 1h were defined as 100% for each strain or isolate. Arithmetric means of at least four runs per sample were calculated. Standard error values are indicated.

enhanced chemiluminescence and electronic imaging technique. Banding signal intensities of PrP^{Sc} after digestion with PK for 1 h are defined as 100%, in order to take account of possible interference effects of PK with other co-purified cellular proteins.

The residual PrP^{Sc} signal after 6 h of proteinase K exposure gives the most useful information when comparing PrP^{Sc}s from different strains or origins.

5. General Comments

The molecular analysis of PrP^{Sc} by immunochemical techniques has allowed the characterization of prion strains, without the need for large-scale mouse inoculation experiments. Many prion strains/isolates can readily be discriminated by PrP^{Sc} glycotyping and molecular mass analysis. Another technique for the discrimination of prion strains is the analysis of long-term proteinase K resistance of PrP^{Sc}. In all these studies, the characteristics of PrP^{Sc} are examined by immunoblots to visualize the non-, mono-, and diglycosylated fractions following PK treatment. However, a variety of experimental factors may influence the results obtained in these experiments. The following rules should therefore be followed, if the interpretation of results on the immunochemical characteristics of PrP^{Sc} is to be reliable.

- All experiments should be performed under standardized conditions, and even minor changes in the experimental setup should be avoided as much as possible.
- Because the accuracy of an immunoblot detection may be affected by a variety of factors, substantial differences between PrP^{Sc} profiles from a given sample may be seen, even if all the above mentioned precautions are applied. Such deviations can only be standardized by repeated testing. In order to obtain representative results, each sample should therefore be electrophoresed and immunoblotted at least 4× and the overall result calculated on the basis of these individual results.
- Glycoform profiles of PrP^{Sc} derived from a given host species may only be comparable when they are obtained using the same antibody.
- Glycoform profiles of PrP^{Sc} derived from different host species may not be comparable, because detection antibodies can have a higher affinity for particular glycoforms of some species.

Taken together, PrP^{Sc}-based typing techniques can provide valuable information for the characterization of TSE cases, and for understanding molecular mechanisms involving different prion strains. Combined with the data on lesion profiles and incubation times in mice (as determined by the Neuropathogenesis Unit in Edinburgh), this information allows a better discrimination and definition of strains.

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6

Differential Targeting of Neurons by Prion Strains

Stephen J. DeArmond

1. Introduction

A basic principle of microbiology that applies to all conventional infectious pathogens is that the disease phenotype is a function of both the infecting agent and the host's response to it. All evidence indicates that this principle is also true for diseases acquired by infection with prions, given that inoculation of different scrapie prion strains into inbred mouse strains shows that reproducible differences in the disease phenotype are determined by both the strain of scrapie prion and a host gene or genes ***(1–6)***. The disease parameters used to characterize and define each prion strain, then and now, include: the relative or complete failure of transmission of a prion strain from one animal species to another, designated the "host species barrier"; incubation time, defined as the time from inoculation of a prion strain to the onset of clinical signs; the neuroanatomic distribution of spongiform degeneration, also designated the "lesion profile"; and whether or not PrP amyloid plaques are formed in the brain. Of these parameters, least is known about how each prion strain targets a different population of neurons for degeneration to create the strain-specific lesion profile. The goal of this report is to review the evidence, which argues that PrP^{Sc} is the main and perhaps sole prion factor determining the disease phenotype, and that PrP^{C} expressed by the host animal is the predominant host factor determining the disease phenotype, including differential targeting of neurons.

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2. Basic Prion Biology

2.1. Prions

Prusiner *(7)* named the infectious agent that transmits scrapie a "prion" when he discovered that it is composed solely of a single, protease-resistant protein, designated the scrapie prion protein, or PrP^{Sc}. Subsequently, it was found that PrP^{Sc} is derived from a constitutively expressed and protease-sensitive mammalian protein designated PrP^{C} ***(8–10)***. Today, a large mass of reproducible data supports the "protein only" hypothesis, which states that a prion is a proteinaceous infectious particle that lacks nucleic acid ***(11)***. The properties of the prion protein that give it the behavioral characteristics of a conventional infectious agent, such as a virus, are: its ability to exist in two conformations, one that is largely α-helical (PrP^{C}) and one that is largely β-sheet (PrP^{Sc}), and the ability of PrP^{Sc} to induce an identical β-sheeted conformation in PrP^{C} and, in doing so, begin a self-perpetuating process that results in increasing prion infectivity titers.

2.2. Nascent PrP^{Sc} Is Derived from the Host's PrP^{C}

Diverse, reproducible data indicate that the PrP^{Sc}, which comprises an infecting prion, catalyzes the conversion of the host's PrP^{C} to nascent PrP^{Sc} for reviews, (*see* **refs.** ***12*** and ***13***). The factors and steps involved in propagation of prions can be summarized as follows:

1. PrP^{C} and PrP^{Sc} have different conformations: PrP^{Sc} is 43% β-sheet and 30% α-helix, whereas PrP^{C} is 3% β-sheet and 42% α-helix ***(14,15)***;
2. Formation of nascent PrP^{Sc} requires synthesis of PrP^{C} by the host and transport of PrP^{C} to the cell surface, since blocking its export from the endoplasmic reticulum-Golgi complex to the plasma membrane inhibits formation of PrP^{Sc} ***(16)***, since exposure of scrapie-infected cells to phosphatidylinositol-specific phospholipase C (PIPLC), which releases PrP^{C} from the cell surface, also inhibits formation of PrP^{Sc} ***(17)***, and since PrP^{Sc} is not formed in PrP knockout mice exposed acutely or chronically to scrapie prions ***(18–21)***;
3. An infecting PrP^{Sc} binds to PrP^{C} ***(22–24)***, a step which appears to require binding of PrP^{C} to a host factor, provisionally designated "protein X" ***(24,25)***;
4. The conformation of PrP^{Sc} is replicated precisely in PrP^{C} during the latter's conversion to nascent PrP^{Sc} ***(26,27)***;
5. PrP^{Sc} accumulates in the brain, because it is protease-resistant; PrP^{C} maintains a steady-state concentration in the brain, because it is degradable and its rate of synthesis is equal to its rate of degradation.

In the central nervous system, it is currently assumed that these steps occur in caveolae-like domains (CLD) of neuronal plasma membranes, because 90% of PrP^{C} and PrP^{Sc} are localized to caveolae-like domains ***(28)***.

2.3. The Conversion of PrP^{C} to Nascent PrP^{Sc}, not Only the Presence of PrP^{Sc}, Is a Requirement for Neurodegeneration

Both acute and chronic exposure of the brain in PrP knockout mice to prions have failed to result in propagation of prions or to cause neuropathological changes ***(18–21)***. This indicates that exogenously derived PrP^{Sc} by itself is not pathogenic; rather, in order for PrP^{Sc} to cause neuronal degeneration, it must be derived from PrP^{C}. It is likely that nascent PrP^{Sc} derived from glycolipid anchored PrP^{C} enters a cellular compartment, where it can disrupt functions that PrP^{Sc} in the extracellular space cannot. It is possible that PrP^{Sc} itself must be glycosylphosphatidylinositol (GPI)-anchored to membranes, to be pathogenic, and that it can only do so if it is derived from GPI anchored PrP^{C}. A corollary to these findings is that the conversion of PrP^{C} to PrP^{Sc} must occur in neurons, in order for PrP^{Sc} to cause neuronal dysfunction and degeneration.

2.4. PrP^{Sc} Conformation Encodes Prion Strain Behavior

Persuasive evidence that the prion factor that gives it strain-like properties is the three-dimensional conformation of its PrP^{Sc} has come from transmissions of familial Creutzfeldt-Jakob disease (CJD) cases into transgenic (Tg) mice expressing a chimeric mouse-human-mouse (MHu2M) PrP transgene ***(27)***. In familial fatal insomnia while that is genetically linked to a mutation of *PRNP* gene at codon 178, FFI(D178N), the protease-resistant fragment of PrP^{Sc} after deglycosylation, migrates as a single 19-kDa fragment, which from familial CJD linked to a mutation at codon 200, fCJD(E200K), is 21 kDa. Subsequently, the difference in molecular size was found to result from different degrees of proteolytic cleavage of the N-termini of these human PrP^{Sc} molecules ***(29)***. The reproducibility of the size differences argues for different stable molecular conformations that protect different lengths of the N-terminus from proteolysis. It is not surprising that mutated (mu) PrP^{Sc}s, with different amino acid substitutions, should have different molecular conformations, because their amino acid sequences are different. The question remained whether those different conformations could be transferred to any PrP^{C}.

Chimeric mouse-human-mouse PrP^{C} in which the amino acid sequence between residues 80 and 150 is identical to that in humans and the sequences on either side are identical to those in mice, PrP^{C}(MHu2M), is readily converted to PrP^{Sc}(MHu2M) by human prions ***(24)***. On the first passage of FFI(D178N) prions to Tg(MHu2M) mice, incubation times were about 206 d,

and the most intense vacuolation was confined to the thalamus; in contrast, incubation times in Tg(MHu2M) mice inoculated with fCJD(E200K) prions were about 170 d, and the most intense vacuolation and PrP^{Sc} deposition were widespread throughout the cerebral hemispheres and brainstem (**Fig. 1**; *see **ref. 27***). Moreover, Western analysis of the deglycosylated, protease-resistant portions of the PrP^{Sc}s in the mouse brain homogenates migrated to 19 kDa for FFI and 21 kDa for fCJD(E200K), like the respective human PrP^{Sc}s.

On the second sequential passage of FFI prions in Tg(MHu2M) mice, incubation time was reduced to about 130 d, vacuolation and PrP^{Sc} accumulation patterns remained the same, and the same 19-kDa PrP^{Sc} digestion product was found. Similarly, the second passage of fCJD(E200K) remained about 170 d, vacuolation and PrP^{Sc} accumulation patterns remained the same, and the same 21-kDa PrP^{Sc} digestion product was found. The persistence of the 19-kDa and 21 kDa PrP fragments from the human brain, and through two sequential transmissions in Tg(MHu2M), supported the idea that the different tertiary conformations of the respective FFI and fCJD prions remained unchanged, even with a change in amino acid sequence from mutated $HuPrP^{C}$s to the chimeric PrP^{C}(MHu2M) in the transgenic mice.

These results argue that PrP^{Sc} conformation differences are a prion strain-specific property and are independent of PrP^{C}'s amino acid sequence. The results also imply that PrP^{Sc} acts as a template for the conversion of PrP^{C} to nascent PrP^{Sc} and, in so doing, imparts a conformation to the nascent PrP^{Sc} that determines the size of the protease resistance fragment of PrP^{Sc}. Finally, the different reproducible vacuolation and PrP^{Sc} deposition profiles and incubation times indicate that two different human prion strains are formed in the human brain as the result of the D178N and E200K *PRNP* mutations.

More recently, we identified two cases of sporadic fatal insomnia (SFI), in which the clinical and neuropathological features were identical to those in FFI, except there was no family history of such a neurodegenerative disorder, and no *PRNP* gene mutation was present in the patient's *PRNP* gene ***(30)***. Deglycosylated, protease-resistant PrP^{Sc} in these cases, also migrated at 19 kDa, as in FFI, and differently than sporadic and familial CJD cases, which migrated at 21 kDa. The same PrP migration patterns were identified in the brains of Tg(MHu2M) mice inoculated with SFI, and the vacuolation histograms and PrP^{Sc} deposition pattern were virtually identical to those found for FFI (**Fig. 1**). These results support the view that, from the perspective of the prion, the clinical and neuropathological disease phenotypes are determined more by the conformation of PrP^{Sc} and less by its amino acid sequence. Furthermore, the PrP^{Sc} conformation, which causes the fatal insomnia can arise as the result of an inherited D178N *PRNP* mutation, or can arise spontaneously. The SFI and FFI phenotypes occur rarely in

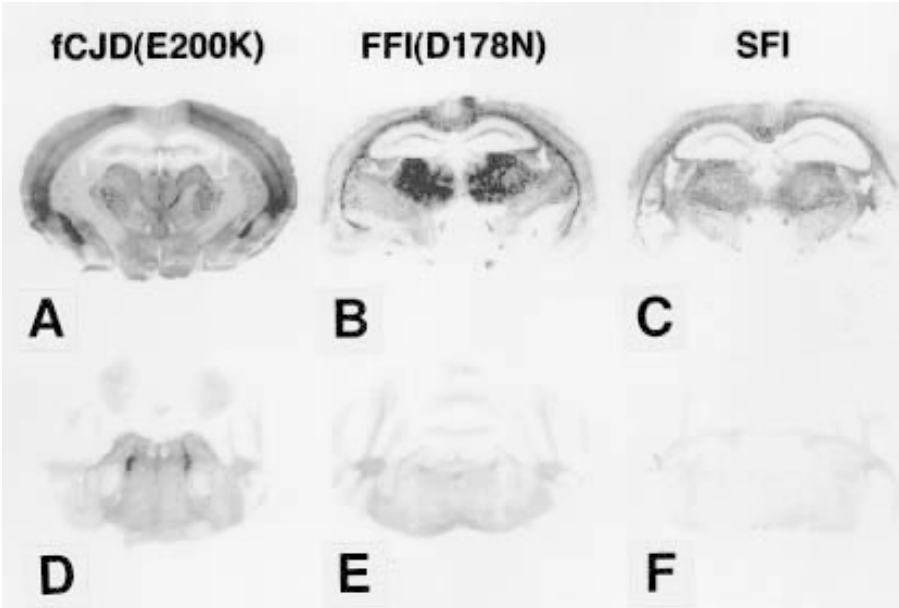

Fig. 1. The regional distribution of PrP^{Sc} in Tg(MHu2M)/$Prnp^{o/o}$ mice inoculated with brain extracts from fCJD(E200K), is different than that caused by inocula from FFI(D178N) and SFI. Histoblots of coronal brain sections at the level of the thalamus and hippocampus (**A**,**B**,**C**) and transverse sections of the upper pons-lower midbrain (**D**,**E**,**F**) were immunostained specifically for protease-resistant PrP^{Sc}. (A,D), fCJD(E200K); (B,E), FFI(D178N); and (C,F) SFI. Adapted from Telling et al. ***(24)*** and Mastrianni et al. ***(30)***.

humans, compared to the number of sporadic and familial CJD phenotypes. It is possible that SFI is caused by an acquired D178N mutation of the *PRNP* gene in a single neuron from which sufficient PrP^{Sc} is formed with the fatal insomnia conformation to trigger the self-perpetuating conversion of wild-type PrP^{C} to nascent PrP^{Sc}.

2.5. PrP^{Sc} Is the Sole Functionally Relevant Component of the Prion

Because of the difficulty eliminating contaminants from purified prions, biochemical enrichment of prions alone cannot completely exclude the possibility that prions contain another functionally important factor ***(31,32)***. The strongest argument in favor of the hypothesis that PrP^{Sc} is the sole component of the prion is whether or not all characteristics of prion diseases and prion propagation can be explained solely by the PrP^{Sc} component. The evidence that this is true comes mostly from studies of prion propagation in Tg mice expressing different PrP constructs. Tg mice serve the dual function of a biological system

in which PrP^{Sc} and PrP^{C} interactions can be examined by manipulating their amino acid sequences, and as a bioassay of the resulting disease phenotype from which prion strain identification can be inferred.

In Tg mice, the host species barrier was found to result from a relatively large difference in the amino acid sequence between PrP^{Sc} comprising the infecting prion, which is determined by the animal from which it was derived, and PrP^{C} expressed by the host animal ***(22,33)***. Further support for this concept is the failure of transmission of any prion strain in Prnp gene knockout mice ($Prnp^{o/o}$ mice) ***(18–21)***. Scrapie incubation times in mice appear to be determined, at least in part, by two polymorphisms of the *Prnp* gene at codons 108 and 189 in inbred mouse strains which result in synthesis of either PrP^{C}-A or PrP^{C}-B ***(34,35)***. These small amino acid sequence differences in the host's PrP^{C} appear to be sufficient to affect its interaction with PrP^{Sc}, the rate of its conversion to nascent PrP^{Sc}, and/or the rate of PrP^{Sc}'s accumulation in the brain, and to account for long and short incubation times. Furthermore, transgenic and congenic mice expressing different proportions and levels of PrP^{C}-A and PrP^{C}-B have shown that scrapie incubation time is inversely proportional to the level of allotype expression ***(36)***.

The homotypic interaction of PrP^{Sc} with PrP^{C}, which initiates the conversion of PrP^{C} to nascent PrP^{Sc}, appears to involve sequence homology of a relatively small domain of the PrP molecule. This was first discovered in Tg mice expressing chimeric SHa and mouse (Mo or M) PrP^{C} in the absence of wild-type $MoPrP^{C}$ ***(23)***. Two chimeric PrP constructs were made on the MoPrP background, one containing two SHaPrP-specific amino acid substitutions at residues 108 and 111, designated MHM2, and the other containing three additional SHaPrP substitutions at residues 138, 154, and 169, designated MH2M. Three Tg mouse lines expressing the former construct, $Tg(MHM2)Prnp^{o/o}$ mice, were resistant to SHa(Sc237) prions similar to non-Tg mice; however, all Tg mice expressing the transgene with five SHa amino acid substitutions, $Tg(MH2M)Prnp^{o/o}$ mice, became clinically ill with SHa(Sc237) prions. It was concluded that 100% homology is not necessary to overcome the host species barrier, and that the PrP domain between residues 90 and 160 is particularly important for PrP^{Sc} and PrP^{C} dimerization (**Fig. 2**).

The relevance of the 90-160 domain for successful interaction of human prions with the mouse's PrP^{C} was also demonstrated in Tg mice expressing the chimeric PrP^{C}(MHu2M) construct, in which residues 90–160 contain the human sequence, but is flanked on both sides by mouse sequences ***(24)***. The PrP 90–160 peptide is highly amyloidogenic, because it has the propensity to form β-sheet bonds with other PrP90–160 peptides and, in doing so, to polymerize into the massive deposits of PrP amyloid characteristic of Gerstmann-Sträussler-Scheinker (GSS) syndromes ***(37)***. This tendency to form

intermolecular β-sheet bonds is facilitated by some amino acid substitutions within and outside the 90–160 region. Thus, in GSS pedigrees, the N-terminus and C-terminus of mutated PrPs is highly truncated: The amyloidogenic PrP peptide in GSS(P102L) consists of residues from about 90 to 170; in GSS(A117V), residues 81–146; in GSS(F198S), residues 58 to 150; in GSS(F198S), residues 81–150; and GSS(Q217R), residues 81–146. As with all amyloids, these peptides polymerize into straight filaments, with β-sheet molecular interactions. The latter finding is one of the arguments that the amyloid phenotype in prion diseases is a function of the amino acid sequence of PrP^C expressed by the host gene.

3. Selective Targeting of Neurons by Prion Strains in Transgenic Mice Expressing Glycosylation Site Mutants

The host factors involved in selective targeting of neurons for neurodegeneration by prion strains are poorly understood. Several lines of evidence indicate that, like the propagation of prions, selective neuronal degeneration is also related to the conversion of PrP^C to PrP^{Sc}. Thus, vacuolar degeneration of neurons, and reactive astrocytic gliosis in a brain region follow the local increase in PrP^{Sc} concentration, and colocalize precisely with sites of PrP^{Sc} deposition ***(38–41)***. A correlation between mutated PrP and neuropathological changes has not yet been established for familial prion diseases of the GSS-type, because nonamyloid plaque PrP in the neuropil is relatively protease-sensitive ***(42)*** and because it may have a transmembrane topography that requires specialized techniques to quantify ***(43)***. Consistent with the neuroanatomic correlation between sites of vacuolar degeneration and PrP^{Sc} deposition in prion diseases acquired by infection, we and others have found that the neuroanatomic pattern of PrP^{Sc} accumulation in the brain is characteristic of each prion strain, and is itself a strain-defining phenotypic parameter (**Fig. 1**) ***(40,44,45)***. These and other observations outlined above have led to the unifying hypothesis that the propagation of prions and neurodegeneration in prion diseases are both linked to the conversion of PrP^C to PrP^{Sc}.

In the context of the steps involved in the conversion of PrP^C to nascent PrP^{Sc}, we hypothesized that selective targeting of neuronal populations in the CNS may be determined by cell-specific differences in the affinity of PrP^{Sc} for PrP^C, which in turn determines brain region differences in the rate of nascent PrP^{Sc} formation. Since PrP^C is glycosylated at Asn residues 181 and 197 (**Fig. 2**) ***(46,47)***, and since Asn-linked oligosaccharide side chains are known to modify the conformation and interaction of glycoproteins ***(48)***, it seemed reasonable to postulate that variations in PrP^C's CHOs may alter the

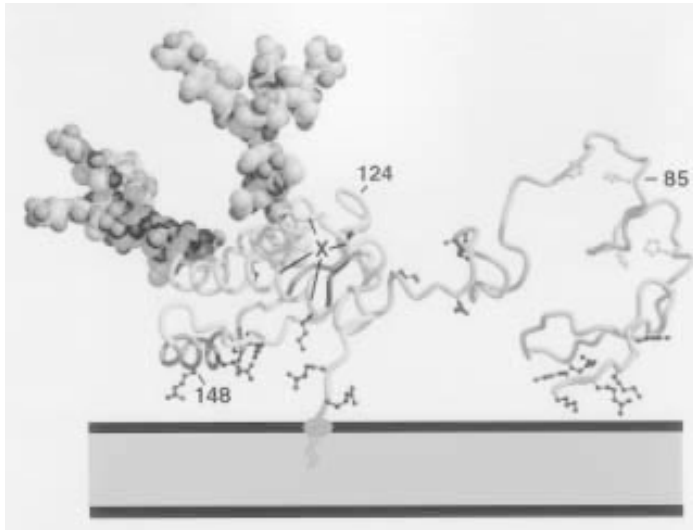

Fig. 2. Structural model of the SHaPrP^{C} molecule. The purpose of the model is to depict the relative sizes of and locations of the Asn-linked oligosaccharides relative to the published structure of SHaPrP fragments inferred from NMR spectroscopy ***(56,57)***. SHaPrP^{C} is shown attached to the plasma membrane by its GPI anchor, to indicate how the range of movement of the N-terminal half of the molecule might be constrained in vivo. A growing body of evidence suggests that the normal physiological role of PrP^{C} is to store and present copper to cells ***(78)***. Recombinant SHaPrP(29–231) binds two Cu^{++} molecules to His residues in the octarepeat region near the N-terminus, and, in doing so, brings about a conformational change ***(79)***. The model was generated by V. Daggett and D. Alanso from the Department of Medicinal Chemistry, University of Washington, Seattle, and is adapted with permission from ref. ***(52).*** The more ordered portion of the molecule, residues 125–231, contains helices B (residues 172–193) and C (200–227). A disulfide bridge links Cys^{179} of helix B with Cys^{214} of helix C (space-filling group). The Asn-linked CHO at residue 181, which is attached to helix B, and the CHO at Asn 197, which is on the bridging peptide between helix B and C, represent the predominant defucosylated moieties present in PrP^{Sc} reported by Endo et al. ***(46)***. The putative protein X-binding sites are indicated with an "X," with lines pointing to the discontinuous epitope on helices C and B, with which it interacts ***(25)***. The disordered portion of the molecule, residues 23-160, was modeled in a random conformation. Whether or not helix A (residues 144–157) exists is debatable. Constraints were applied to the putative Cu^{++} binding histidine residues (ringed side chains) near the N-terminus to bring them into proximity.

size of the energy barrier that must be traversed during formation of PrP^{Sc}. If this is the case, then regional variations in CHO structure could account for formation of PrP^{Sc} in particular areas of the brain. To test this hypothesis, we constructed Tg mice that express SHaPrPs mutated at either or both of the glycosylation consensus sites ***(49)***.

3.1. Expression of Glycosylation-Site Mutant SHaPrP^C in Transgnic Mice

Asn-linked oligosaccharides are attached to Asn residues 181 and 197 in $SHaPrP^C$. To delete one or both of these CHOs, the threonine residues were mutated to Ala within the NXT consensus sequence sites ***(50)***. Single and double glycosylation site mutations were expressed in Tg mice deficient for MoPrP ($Prnp^{o/o}$). The distribution of mutant $SHaPrP^C$ was analyzed by the histoblot technique ***(51)***. Here, analysis is confined to the hippocampus.

Wild-type $SHaPrP^C$ was confined almost exclusively to the dendritic tree region of the CA1–CA4 regions of Ammon's horn and of the dentate gyrus (**Fig. 3A**). It was absent from the nerve cell bodies of the pyramidal and granule cell layers in the respective regions; additionally, it was mostly absent from white matter tracts, such as the corpus callosum that overlies the hippocampus. In contrast, mutation of either one or both glycosylation consensus sites had a profound effect on the anatomical distribution of $SHaPrP^C$. Mutation of the first glycosylation site alone, or in combination with mutation of the second site, resulted in low brain levels of mutated $SHaPrP^C$(T183A) and $SHaPrP^C$(T183A, T199A), accumulation of the respective mutated $SHaPrP^C$s in nerve cell bodies, and little or none in the dendritic trees (**Fig. 3B, C**). When the second glycosylation site was mutated and the first left intact, the brain levels of $SHaPrP^C$(T199A) were about the same as wild-type $SHaPrP^C$. $SHaPrP^C$(T199A) was distributed to all neuronal compartments including nerve cell bodies, the dendritic tree, and axons of the white matter (**Fig. 3D**). These results suggest that the CHO at residue 181 is required for trafficking of PrP^C out of the nerve cell body and into nerve cell processes.

3.2. Transmission of Scrapie to Transgenic Mice Expressing Mutant SHaPrPs

To examine the effects of mutations of the consensus sites for Asn-linked glycosylation on the scrapie phenotype, Tg mice expressing the mutant $SHaPrP^C$s were inoculated with either the Sc237 or 139H hamster-adapted prion strains. Of the Tg mice expressing the three different mutant PrPs, only Tg(SHaPrP-T199A) mice were capable of forming PrP^{Sc}. Two lines of Tg(SHaPrP-T199A)$Prnp^{o/o}$ mice developed signs typical of scrapie, about 550

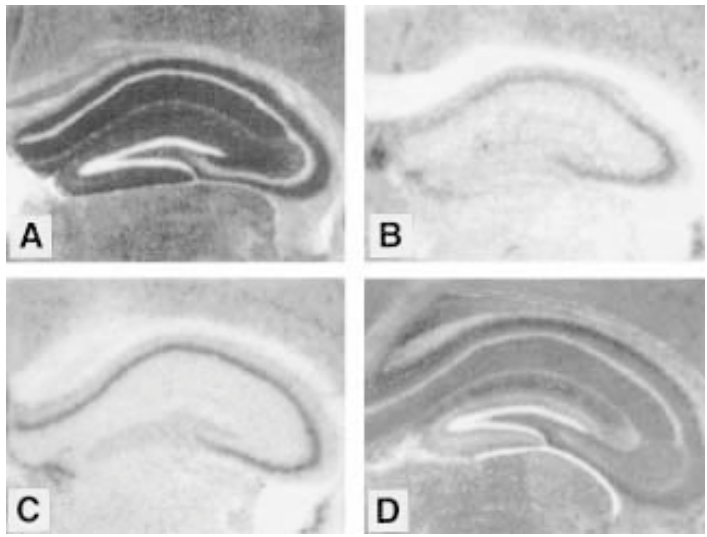

Fig. 3. Mutations of the Asn-linked glycosylation consensus sites alter the distribution of $SHaPrP^{C}$ in the brains of Tg mice: (**A**) Wild-type $SHaPrP^{C}$; (**B**) $SHaPrP^{C}$(T183A); (**C**) $SHaPrP^{C}$(T183A, T199A); and (**D**) $SHaPrP^{C}$(T199A). Histoblots of coronal sections of the hippocampal regions, from normal, uninfected animals, were stained specifically for protease-sensitive PrP^{C} by the histoblot technique. Adapted with permission from ***ref. 49***.

d postinoculation with Sc237, but not with 139H. In contrast, incubation times were about 55 d in Tg(SHaPrP)/$Prnp^{o/o}$ mice expressing levels of wild-type $SHaPrP^{C}$, comparable to those expressing mutant $SHaPrP^{C}$ in Tg(SHaPrP-T199A)$Prnp^{o/o}$ mice. These findings suggest that deletion of the of the CHO at Asn187 created a host species barrier to 139H prions, and also resulted in a 10-fold increase in Sc237 incubation time.

The neuroanatomic distribution of protease-resistant mutant $SHaPrP^{Sc}$(T199A) accumulation in two Tg(SHaPrP-T199A)$Prnp^{o/o}$ mouse lines, and the distribution of protease-resistant wild-type $SHaPrP^{Sc}$ in two Tg(SHaPrP)/$Prnp^{o/o}$ mouse lines, were compared ***(22,33)***. Distributions of wild-type $SHaPrP^{Sc}$ in the two Tg(SHaPrP)/$Prnp^{o/o}$ lines were similar, and the distributions of mutant $SHaPrP^{Sc}$ in the two Tg(SHaPrP-T199A)$Prnp^{o/o}$ were similar; however, the distributions of wild-type $SHaPrP^{Sc}$ and mutant $SHaPrP^{Sc}$ were markedly different (**Fig. 4**). One of the chief differences was little or no mutant $SHaPrP^{Sc}$ in the thalamus and habenula.

These results reveal; that deletion of the CHO at Asn197 has a profound effect on the PrP^{Sc} distribution phenotype, and therefore, on the lesion profile.

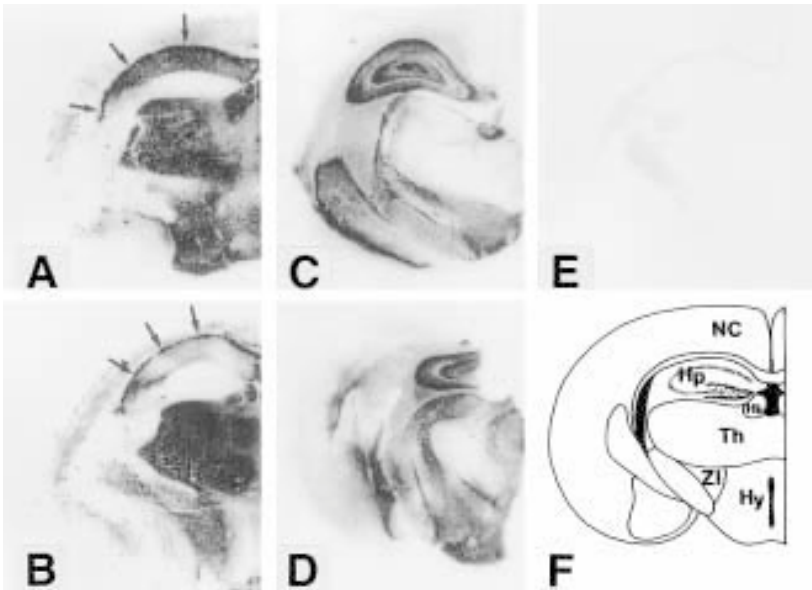

Fig. 4. The regional distribution of wild-type SHaPrP^{Sc} (**A,B**) is markedly different than mutant SHaPrP^{Sc}(T199A) (**C,D**) in Tg mice inoculated with Sc237 prions. Histoblots of coronal sections through the thalamus and hippocampus were immunostained for protease-resistant PrP^{Sc}. (A and B) Two different Tg mouse lines expressing wild-type SHaPrP^{C}. (C and D) Two different Tg mouse lines expressing SHaPrP^{C}(T199A). (E) Tg(SHaPrP -T199A)/Prnp$^{o/o}$ mice inoculated with the 139H scrapie prion strain did not develop signs of scrapie nor form protease-resistant PrP^{Sc}; only weak, nonspecific staining of white matter is seen. Arrows in (A) and (B) point to sites of PrP amyloid plaque formation. (**F**) Diagram: Hb, habenula; Hp, hippocampus; Hy, hypothalamus; NC, neocortex; ZI, zona incerta. Adapted with permission from ***ref. 49***.

Moreover, the PrP amyloid plaque phenotype was also altered. Amyloid plaques composed of wild-type SHaPrP^{Sc} were easily identified in both Tg(SHaPrP)/Prnp$^{o/o}$ lines by their characteristic subcallosal location and size (**Fig. 4**), as reported earlier ***(22)***; however, no PrP amyloid-like deposits were identified in Tg(SHaPrP-T199A)Prnp$^{o/o}$ mouse lines. This implies that deletion of CHO at Asn197 results in a poorly amyloidogenic SHaPrP^{Sc}. In summary, deletion of the Asn-lined CHO at residue 197 affected all of the prion strain related phenotypic parameters: the host species barrier, scrapie incubation time, the PrP^{Sc} distribution pattern in the brain (and, therefore, the lesion profile), and whether or not PrP amyloid forms.

4. PrP^C Glycoform Heterogeneity as a Function of Brain Region

To explain selective targeting of different neuronal populations by prion strain, we hypothesized that there are cell-specific differences in the affinity of an infecting PrP^{Sc} for PrP^C, which in turn determines brain region differences in the rate of nascent PrP^{Sc} formation and accumulation. From the evidence described above, it seemed reasonable to postulate that neuron-specific variations in the complex CHOs may alter the interaction between an infecting PrP^{Sc} and a neuron's PrP^C, or the size of the energy barrier that must be traversed during formation of nascent PrP^{Sc}. The goal of our next studies was to comprehensively test whether or not brain regions in Tg mice and Syrian hamsters (SHa) synthesize different sets of $SHaPrP^C$ glycoforms, as inferred from 2-D gel electrophoresis patterns ***(52)***.

4.1. Charge Isomers in Transgenic Mice Expressing Glycosylation Site Mutant PrP

Before beginning studies of SHas, we tested whether or not PrP^C charge isomers vary as a function of the number of CHOs attached to PrPs in Tg mice expressing wild-type and glycosylation mutant $SHaPrP^C$s ***(49)***. Earlier studies had shown that PrP charge isomers resulted from variable sialylation of Asn-linked CHOs ***(46,47,53)***. A large number of charge isomers were found with wild-type $SHaPrP^C$; but, mutation of both Asn-linked glycosylation consensus sites reduced the number of isomers to one major and two minor spots (**Fig. 5A, D**). An intermediate number of charge isomers were found when one of the two consensus sites was mutated to produce either $SHaPrP^C$(T183A) or $SHaPrP^C$(T199A) (**Fig. 5B, C**).

4.2. Regional Differences in the Number and Location of PrP^C Isoelectric Points in SHa Brain

In preliminary 1-D gel electrophoresis studies, the relative concentration of wild-type $SHaPrP^C$ was found to vary as a function of brain region in SHa brain. These differences were sufficient to influence the number of isoelectric points visible on Western transfers. Specifically, a progressive loss of $SHaPrP^C$ charge isomers occurred on the acidic side of 2-D gels which was proportional to the amount of $SHaPrP^C$ loaded. Therefore, the author adjusted the amount of sample loaded on the gel from each brain region, to equalize the amount of $SHaPrP^C$. When equal amounts of $SHaPrP^C$ from each brain region were analyzed, differences in the number and locations of $SHaPrP^C$ isoelectric points could be identified by visual inspection (**Fig. 6**). To determine whether or not

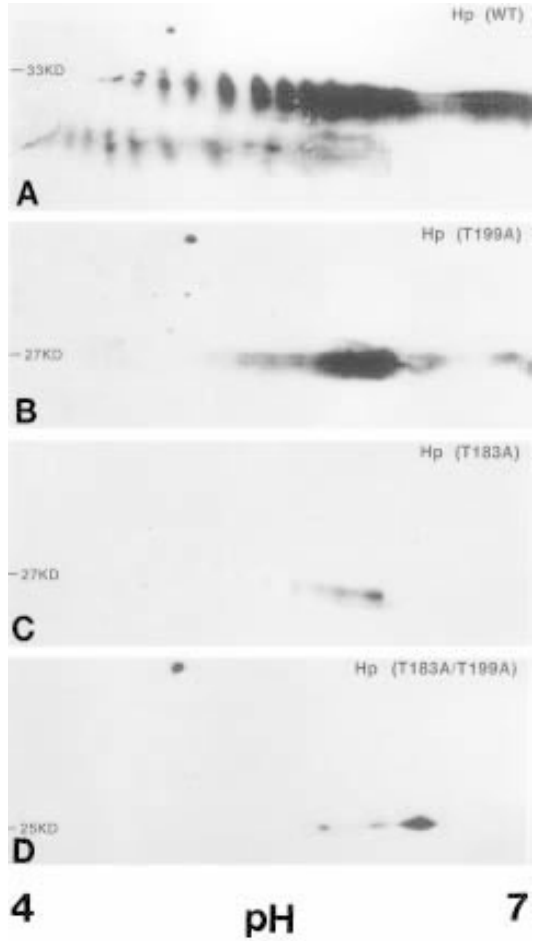

Fig. 5. The majority of PrP^C charge isomers originate from its two Asn-linked oligosaccharides. Wild-type $SHaPrP^C$ and deglycosylation mutant $SHaPrP^C$s expressed in hippocampus (Hp) of Tg mice are compared. (**A**) Wild-type $SHaPrP^C$; (**B**) $SHaPrP^C$(T199A); (**C**) $SHaPrP^C$(T183A); (**D**) $SHaPrP^C$(T183A, T199A). Western transfers of 2-D gels were immunostained with the 13A5 monoclonal antibody. Adapted with permission from ***ref. 49.***

isoelectric patterns for a brain region were reproducible and unique between experimental runs and among different groups of animals, homogenates from 2–4 groups of hamsters were compared, using two ampholyte ratios (ampholyte ratios were created using Bio-Lyte ampholytes from Bio-Rad, as follows: ratio A, BioLyte 3/10:BioLyte 5/7 = 1:2 and ratio B, BioLyte 3/10:BioLyte 5/8 = 1:4). The greatest number of comparisons were made for the neocortex, hippocam-

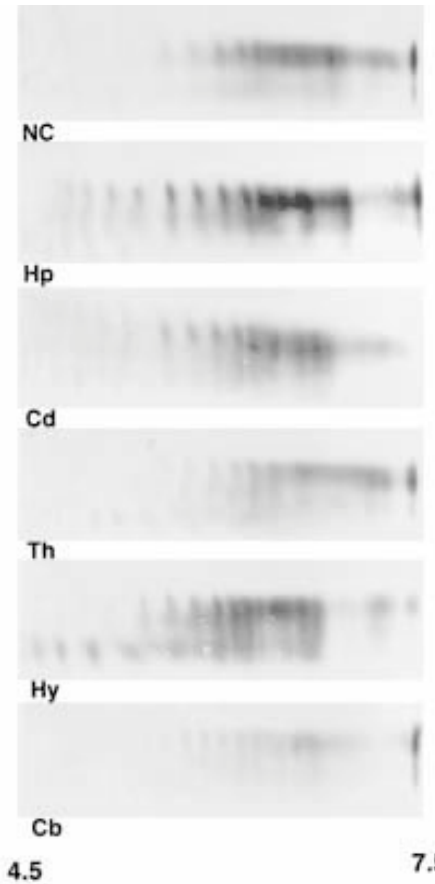

Fig. 6. Differences in the PrP^C isoelectric point patterns from six brain regions are readily detectable by visual inspection of Western transfers immunostained with the 3F4 mAb. Equal amounts of PrP^C from each region were analyzed. Approximate pH range is indicated at bottom. Hp, hippocampus; Cb, cerebellum; Th, thalamus; Hy, hypothalamus; Cd, head of caudate nucleus; NC, frontoparietal neocortex. Adapted with permission from ***ref. 52***.

pus and cerebellum because there were sufficient quantities of homogenate from these regions to perform multiple electrophoresis runs.

To assemble all data from multiple electrophoresis runs, and from multiple animal groups, into a single graph, the isoelectric points from each run were normalized by choosing one spot that could be recognized, and whose pH was known. An isoelectric point between pH 5.8 and 5.9 was present in each run and animal group, and served this purpose well for the two ampholyte ratios used in this study. The distance to that spot from the alkaline end of each elec-

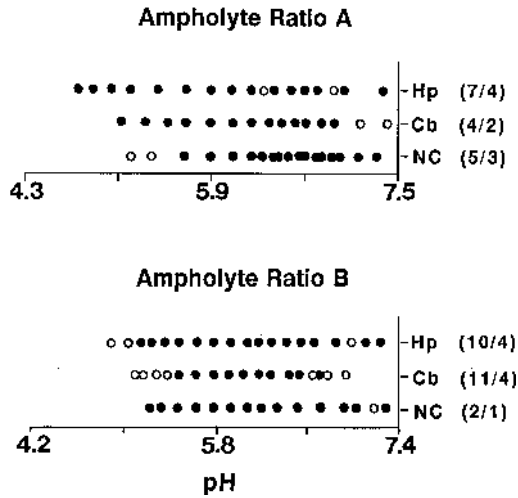

Fig. 7. Graphic summary of multiple 2-D electrophoresis runs for the neocortex (NC), hippocampus (Hp), and cerebellum (Cb). Isoelectric points, which appeared in every run from each group of animals, are represented by solid circles, and those that failed to occur one or more runs are represented by open circles. The number of electrophoresis runs (left-hand number) and the number of animal groups studied (right hand number) are indicated in parentheses. Adapted with permission from ***ref. 52***.

trophoresis pattern was measured, and the fractional distance traveled by other spots calculated (relative isoelectric mobility) (**Fig. 7**). Analysis of isoelectric points in this way revealed patterns common to each brain region, as well as differences. The isoelectric point at pH 5.8–5.9 was located between another, more acidic point and two more alkaline points. These four isoelectric points and the spacing between them, were similar in all brain regions, including those not shown in Fig. 7. Differences that distinguished each brain region were on both the acidic and alkaline side of the four points. The more acidic isoelectric points resolved into distinct spots; the more alkaline points between pH 6.3 and 7.3 tended to merge together (**Fig. 6** and **7**). The latter was particularly characteristic of the neocortex with ampholyte ratio A.

The isoelectric point patterns from multiple runs were consistently different for the neocortex, hippocampus and cerebellum. Most of the isoelectric points were present in each run (constant points); however, a few isoelectric points were found in some runs but not others (non-constant points) (**Fig. 7**). For ampholyte ratio A, the hippocampus had 15 constant isoelectric points ranging in pH from 4.8 to 7.36 ($N = 7$) whereas the cerebellum had 13, ranging from pH 5.15 to 7.33 ($N = 4$). For ampholyte ratio B, the hippocampus had 16 constant isoelectric points from pH 5.18 to 7.25 ($N = 10$), and the cerebellum, 10

constant points from pH 5.51 to 6.75 ($N = 11$). 2-D gel runs of neocortical homogenates from three groups of animals were different than both the hippocampal and cerebellar patterns, particularly with ampholyte ratio A. One striking difference was the number of constant isoelectric points between pH 6.1 and 7.1, which was estimated to be 12 for the neocortex, 7 for the hippocampus (plus 2 inconstant points), and 8 for the cerebellum. These results indicate that the majority of isoelectric points are reproducible from run to run and between animals, and are, therefore, characteristic of a brain region.

4.3. PrP^C Is Similar to Other GPs

The results of our studies show heterogeneity of PrP^C charge isomers as a function of brain region. Moreover, because our earlier study ***(49)***, and those of others ***(46,47,53)***, found that virtually all of PrP^C's isoelectric points originate from its two Asn-linked CHOs, the charge heterogeneity indicates that there are reproducible brain region differences in the structure of PrP^C's CHOs. Cell-type-specific glycosylation patterns are well known for other glycoproteins, so that they are referred to as a cell's "glycotype" ***(54)***. The results with PrP^C are, therefore, consistent with what is known about cell-specific characteristics of other glycoproteins. Finding PrP^C glycotype variations as a function of brain region is consistent with our hypothesis that selective targeting of neurons in prion diseases is modulated, at least in part, by PrP^C's CHOs.

Because PrP^C from dissected brain regions was analyzed, the spectrum of glycoforms in each probably represents the products of multiple nerve cell types. Whether the PrP^C located in a neuroanatomically defined brain region is synthesized by resident neurons, or whether it is carried into the region by axonal transport from neurons outside of the region, or both, is unknown. It is reasonable to assume that glial and endothelial cells in each region contribute little to the set of glycoforms, since they express less than three PrP mRNAs per cell; neurons express from 10 to 50 depending on the nerve cell type ***(55)***. Although prion strains can be identified by reproducible differences in the neuroanatomic pattern of PrP^{Sc} deposition, it is also true that many prion strains target the same brain regions for conversion of PrP^C to PrP^{Sc} ***(40,44,45)***. If indeed PrP^C's Asn-linked CHOs modulate its interaction with PrP^{Sc}, finding only minor differences in the isoelectric point patterns among some brain regions may explain why the same brain regions are targeted by different prion strains.

5. Evidence that PrP^C's Asn-Linked CHOs Probably Influence its Interaction with an Infecting PrP^{Sc}

How might Asn-linked oligosaccharides influence PrP^C's interaction with PrP^{Sc} and be the basis of selective neuronal targeting? One possibility is that

the flexibility and/or conformation of that portion of the PrP^C molecule that interacts with PrP^{Sc}, and whose conformation is changed, during conversion, to nascent PrP^{Sc}, is influenced by variations in PrP^C's CHOs. The PrP molecule has two domains that play different roles in the conversion of PrP^C to PrP^{Sc} (**Fig. 2**). First, there is a stable or ordered core domain that contains the two Asn-linked oligosaccharides; two α-helices, designated helix B and helix C, which are stabilized by a disulfide bridge between cysteine[179] and cysteine[214]; the GPI attached to the C-terminus at residue 231, which anchors PrP^C to the plasma membrane; and the protein X binding sites, which are believed to lower the energy barrier for conversion of PrP^C to PrP^{Sc} when PrP^C binds to protein X ***(24,25)***. Second, there is a variable or disordered, domain that contains the portion of the molecule that interacts with PrP^{Sc} and changes its conformation from primarily unstructured in PrP^C to β-sheet in PrP^{Sc} ***(13)***. Nuclear magnetic resonance and nuclear Overhauser effect spectroscopy of two large synthetic PrP fragments, PrP90-231 (*56*) and PrP29–231 ***(57)***, suggest that the variable domain of PrP^C is mostly unstructured, but may contain a relatively short α-helix (helix A, residues 144–156) and two short antiparallel β-strands (residues 129–131 and 161–163). Investigations of the steps required for prion propagation and neurodegeneration in Tg mice expressing chimeric mouse-hamster-mouse or mouse-human-mouse PrP transgenes indicate that residues 90 to 150 in the variable region play a particularly important role in the interaction of PrP^C with PrP^{Sc} leading to the conversion of the former to the latter ***(23,24)***. Residues 90–150 are largely unstructured or weakly helical in PrP^C ***(56,57)*** but are predicted to be β-sheet in PrP^{Sc} ***(13)***. In addition, putative helix A may be converted to β-sheet, along with other portions of the variable region, during the conversion to PrP^{Sc}. Consistent with these possibilities, Fourier transform infrared and circular dichroism spectroscopy indicate that PrP^C contains about 40% α-helix and about 3% β-sheet; PrP^{Sc} contains about 30% α-helix and 45% β-sheet ***(14)***.

Several lines of evidence indicate that Asn-linked CHOs influence protein conformation ***(48)***. For example, Asn-linked glycosylation was found to cause an hemagglutinin peptide to adopt a more compact, folded conformation ***(58)***. Glycosylation of epitopes in the rabies virus glycoprotein was found to disrupt α-helical structure, and to induce formation of a β-turn; moreover, the most dramatic effects occurred on addition of a single, simple carbohydrate ***(59)***. The latter raises the possibility that some PrP^C glycoforms may more readily convert to PrP^{Sc} than others. Glycosylation appears to influence disulfide bridges between serum immunoglobulin M peptides, possibly by reducing the mobility of the peptide tailpieces ***(60)***. Glycosylation of a highly conserved 15-residue loop region in the nicotinic acetylcholine receptor facilitates disulfide bridge formation, apparently by bringing the termini of the loop into closer

proximity ***(61)***. In some cases, such as the CD2 receptor of T-cells, the oligosaccharide binds to a positively charged cluster of five solvent-exposed lysine residues that destabilize the functionally relevant conformation in the deglycosylated molecule ***(62,63)***. Regarding the influence of oligosaccharides on specific targeting of cells, a single carbohydrate residue difference among cell surface antigens determines whether or not *E. coli* targets the urinary tract for infection ***(64)***. The foregoing provide precedent for the idea that the CHOs may influence the conformation and flexibility of residues 90–150 in the PrP^{C} molecule.

6. Prion Strain Specificity of PrP^{Sc} Glycotypes

Given the evidence that each neuron population synthesizes PrP^{C} with a different complement of CHOs, and the fact that each prion strain targets a different set of neurons for conversion of PrP^{C} to PrP^{Sc}, one would predict that PrP^{Sc} glycoforms are also prion-strain-specific. To date, no laboratory has compared PrP^{Sc} isoelectric patterns for different strains of prions. However, different proportions of di-, mono-, and nonglycosylated PrP27–30 (the proteinase K digestion product of PrP^{Sc}) have been found to characterize human and animal prion strains. For example, a preponderance of diglycosylated PrP27–30 has been found by Western analysis in the central nervous system of patients with new variant CJD of Great Britain, compared with most sporadic and iatrogenic CJD cases ***(65,66)***. Similarly, the proportions of unglycosylated to heavily glycosylated PrP 27–30 following passage of prions in mice, are sufficiently different to differentiate seven scrapie prion strains ***(67)***.

In animal models of scrapie, the vast majority of PrP^{C} molecules that are converted to PrP^{Sc} are probably doubly glycosylated, because deletion of both CHOs, or deletion of the CHO at Asn181 alone, results in retention of PrP^{C} in the cell body, failure of PrP^{C} transport to the plasma membrane of neuritic processes, and rapid degradation of PrP^{C} ***(49)***. Lehmann and Harris ***(68)*** found that $MoPrP^{C}$ in Chinese hamster ovary cells mutated to delete the CHO at Asn 180 alone or to delete both CHOs at Asn180, and Asn196 failed to reach the cell surface after synthesis; in contrast, both wild-type $MoPrP^{C}$ synthesized in the presence of tunicamycin or mutated $MoPrP^{C}$, in which the CHO at Asn196 alone was deleted, were detected on the plasma membrane. These results suggest that the CHO at Asn181 in SHaPrP (or at Asn180, in the case of MoPrP) is particularly important for trafficking of PrP^{C} to the plasma membrane, in general, and to neuritic processes of neurons, in particular. PrP^{C} must reach the cell surface prior to conversion to PrP^{Sc} because blocking PrP^{C} export from the endoplasmi reticulum-Golgi complex to the plasma membrane inhibits formation of PrP^{Sc} ***(16)*** and, because exposure of scrapie-infected cells to phosphatidylinositol-specific phospholipase C which releases PrP^{C} from the

cell surface, also inhibits formation of PrP^{Sc} ***(17)***. Although PrP^{C} that is monoglycosylated at Asn181 (CHO at 197 deleted) is transported to the plasma membrane, it is unlikely that a significant amount of it accumulates as nascent PrP^{Sc}. Thus, although Asn 181 monoglycosylated PrP^{C} has a normal distribution and concentration in the brain that is similar to wild-type PrP^{C}, it requires over 500 d for conversion to PrP^{Sc}, following inoculation with Sc237 prions, which is more than 3× as long as the time to death in the animals expressing wild-type PrP^{C} ***(49)***.

All of the above observations suggest that the variable, but significant, proportions of mono- and nonglycosylated PrP^{Sc} which are characteristic of each prion strain, are probably formed after PrP^{C} is converted to PrP^{Sc}. Postconversion modification of PrP^{Sc}'s CHOs is likely, because PrP^{Sc}'s peptide component is highly protease-resistant; its CHOs, like those of other plasma membrane glycoproteins, are relatively sensitive to glycosidases ***(69,70)***. CHO degradation may occur in lysosomes, where a proportion of PrP^{Sc} becomes stored ***(16,71)***, or PrP^{Sc} molecules may be partially or completely deglycosylated by recycling through nonlysosomal endocytic compartments ***(72)***. What then is the relationship of the neuroanatomic site of formation of PrP^{Sc} to the origin of prion-strain-specific proportions of di-, mono-, and nonglycosylated PrP^{Sc}? It may be that some brain region-specific PrP^{Sc} glycoforms are preferentially trafficked to cellular compartments where CHOs are digested. Furthermore, some PrP^{Sc} glycoforms may be more easily degraded than others. Regarding the last possibility, degradation of renin glycoforms by the liver is directly related to the proportion of acidic isoelectric points ***(73)***. Alternatively, there may be significant differences in the rate or specificity of CHO digestion among neuron populations.

7. Familial CJD(T183A)

A Brazilian family with an autosomal dominant form of CJD was found to have a T183A mutation of the *PRNP* gene ***(74)***, which is predicted to prevent glycosylation of the Asn at residue 181 in human PrP, as it did in our transgenic mouse studies. Nine members of the family were affected by the disorder with a mean age of onset of 44.8 ± 3.8 yr and a duration of 4.2 ± 2.4 yr. Intense vacuolation was largely confined to the deeper layers of the neocortex and to the putamen. Immunoperoxidase staining of formalin-fixed, paraffin-embedded tissue sections for abnormal PrP, by the formic acid and hydrolytic autoclaving methods, showed deposition in the cerebellar cortex and putamen, but not in the neocortex. Nerve cell body immunostaining was not described, and is not obvious in their published photomicrographs.

This human pedigree is interesting, because it is different than the author's findings in Tg mice in which we showed that expression of $SHaPrP^{C}$(T183A),

in the absence of wild-type MoPrP^{C} in Tg(SHaPrP T183A)Prnp$^{o/o}$ mice, does not result in spontaneous neurodegeneration, nor does it make the mouse susceptible to infection with prions. In contrast, the family members expressing mutated HuPrP(T183A) died at a relatively young age with spongiform encephalopathy. The difference between the author's Tg mouse model and the human cases is that, in the latter, mutated HuPrP was expressed concomitantly with wild-type HuPrP^{C}. We believe that Tg(SHaPrP T183A)Prnp$^{o/o}$ mice were immune from disease because mutated SHaPrP(T183A) accumulated at low levels in Tg mouse brains and because of a rapid rate of degradation following synthesis. Also, SHaPrP(T183A) remained confined to nerve cell bodies, and was not transported to neuronal processes, where it would have a chance to interact with an infecting PrP^{Sc}. Concomitant expression of HuPrP(T183A) with wild-type HuPrP^{C} raises the possibility that the former alters the conformation of the latter, converting it to abnormal pathogenic PrP, or that the presence of wild-type HuPrP^{C} prevents degradation of mutated PrP, and allows it to be transported to cellular compartments, where it could cause spongiform degeneration of neurons.

8. PrP^{C} Is the Main Host Factor Determining the Scrapie Phenotype

By viewing prion diseases from the perspective of the neuropathological changes, it has been learned that host-determined variations in PrP^{C} play as important a role in determining the disease phenotype as the conformation of PrP^{Sc} comprising an infecting prion. The role of PrP^{C} in the pathogenesis of prion diseases cannot be overemphasized. Indeed, the critics of the protein-only hypothesis often argue that an infectious agent, composed solely of a single, abnormally folded protein, cannot encode all the information necessary to account for the known variations in the prion disease phenotype. In this, they are probably correct because there are most likely only a limited number of different stable strain determining conformations of PrP^{Sc} possible, excluding contributions of PrP^{Sc}'s CHOs. However, these critics do not differentiate between coding of strain information in prions or the combination of both prion and host factors that generate the disease phenotype. The results of our studies, as well as others reviewed above, indicate there are more than sufficient animal species-determined variations in the amino acid sequence of PrP^{C}, the level of expression of PrP^{C} allotypes, and the CHO structure of PrP^{C}, in combination with variations in the conformation and amino acid sequence of PrP^{Sc} to account for all of the known variations in the prion disease phenotype. Other host factors may also influence the disease phenotype, such as the response of microglia or astrocytes ***(75,76)***; nevertheless, all of the evidence indicates that PrP^{C} is the preeminent host factor.

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7

Transgenic Studies of Prion Diseases

Glenn C. Telling

1. Introduction

This chapter reviews studies that involve the manipulation of prion protein (PrP) genes by transgenesis in mice. These consist of two approaches: PrP gene knockout and gene replacement using homologous recombination in embryonic stem cells; and microinjection of transgenes into fertilized embryos. These studies have provided important insights into the pathogenesis of prion diseases including the molecular basis of prion strains and species barriers. Transgenic approaches have also provided important information about the mechanism by which human prion diseases can be both genetic and infectious. Despite these advances, our understanding of these unique pathogens is far from complete. Transgenic approaches will doubtless remain the cornerstone of investigations into the prion diseases, and will be important in the development of therapeutic agents in coming years**.**

2. Knockout and Gene Replacement Studies

Some of the most compelling evidence to date for the protein-only hypothesis of prion replication derives from experiments with knockout transgenic mice. Because PrP^{C} is the source of PrP^{Sc}, the model predicts that elimination of PrP^{C} would abolish prion replication. To test this, the mouse PrP gene, referred to as *Prnp*, was disrupted by homologous recombination in embryonic stem cells. Stem cells containing the disrupted *Prnp* gene were introduced into mouse blastocysts, and knockout mice were established ***(1–3)***. Unlike wild-type mice, the resultant homozygous null mice, referred to as $Prnp^{0/0}$, which express no PrP^{C}, fail to develop the characteristic clinical and neuropathological symptoms of scrapie after inoculation with mouse prions, and do not propa-

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gate prion infectivity ***(3–6)***, while mice that are hemizygous for PrP gene ablation, and therefore expresses one-half the normal level of PrP^C, have prolonged incubation times ***(5–7)***.

Two *Prnp*$^{0/0}$ lines, in which the PrP coding sequence was disrupted, were independently generated in Zurich and Edinburgh. Contrary to expectation, these mice developed normally, and suffered no gross phenotypic defects ***(1,2)***. These results raised the possibility that adaptive changes that compensate for the loss of PrP^C function occur during the development of *Prnp*$^{0/0}$ mice. To test this hypothesis, transgenic mice were produced in which expression of transgene-expressed PrP^C could be experimentally regulated. Using the tetracycline gene-response system, mice were produced that co-express a tetracycline (TET)-responsive transactivator (tTA), and a tTA-responsive promoter that drives PrP expression ***(8)***. The tTA consists of the TET repressor fused to the transactivation domain of herpes simplex virus VP16, and binds specifically and with high affinity to the TET operator (tetO). Binding of tTA normally activates transcription of the PrP gene, but binding of doxycycline (DOX), a TET analog, to tTA prevents the tTA protein from binding to tetO, which in turn prevents PrP gene expression. Thus, PrP^C is expressed in the absence of DOX, but not in its presence. Repressing PrP^C expression by oral administration of DOX was not deleterious to adult mice. However, since DOX treatment did not completely inhibit PrP^C expression in these mice, it is not clear whether this residual expression masks the true phenotype of *Prnp*$^{0/0}$ mice.

A third line of gene-targeted *Prnp*$^{0/0}$ mice generated in Nagasaki also developed normally, but, unlike the *Prnp*$^{0/0}$ mice created in Zurich and Edinburgh, they showed progressive ataxia and cerebellar Purkinje cell degeneration at about 70 wks ***(9)***. Further histologic studies of these mice also revealed abnormal myelination in the spinal cord and peripheral nerves ***(10)***. These *Prnp*$^{0/0}$ mice were successfully rescued from demyelination and Purkinje cell degeneration by introduction of a transgene encoding wild-type mouse PrP^C, as a result of mating to Tg(MoPrP-A)4053/FVB mice ***(11)***. Resolving the molecular basis of the phenotypic differences in the different *Prnp*$^{0/0}$ lines is important because the results may have widespread applications for understanding general mechanisms of neurodegeneration. The recent discovery of the Doppel (Dpl) locus, referred to as *Prnd*, 16 kb downstream of Prnp provided an important clue to this conundrum ***(12)***. Dpl is upregulated in the central nervous system (CNS) of *Prnp*$^{0/0}$ mice that develop late-onset ataxia, suggesting that Dpl may provoke neurodegeneration.

Several other phenotypic defects have also been investigated in *Prnp*$^{0/0}$ mice including altered circadian rhythms and sleep patterns ***(13)***, alterations in superoxide dismutase activity ***(14)*** and defects in copper metabolism ***(15)***. Electrophysiological studies suggested that γ-aminobutyrio acid A receptor-medi-

ated fast inhibition and long-term potentiation are impaired in hippocampal slices from $Prnp^{0/0}$ mice ***(16,17)***, although these defects could not be identified in other studies ***(18,19)***.

Studies with inbred strains of mice demonstrate that a scrapie incubation time locus in mice, referred to as *Prn-i*, was genetically linked to the mouse PrP gene, *Prnp* ***(20)***. Inbred strains of mice with long and short scrapie incubation periods harbor distinct *Prnp* alleles, referred to as $Prnp^a$ and $Prnp^b$, which differ at codons 108 (Leucine to Phenylalanine) and 189 (Threonine to Valine) ***(21)***. In order to precisely define the effects of these PrP polymorphisms on prion incubation times, the coding sequence of the endogenous $Prnp^a$ gene in embryonic stem cells was replaced with the $Prnp^b$ coding sequence by homologous recombination. Studies with the resulting transgenic mice confirmed, as predicted, that *Prn-i* and *Prnp* are the same gene, and that amino acid differences at residues 108 and/or 189 in PrP-A and PrP-B modulate scrapie incubation times ***(22)***. A mutation equivalent to P102L in the human PrP gene, associated with Gerstmann-Sträussler Scheinker disease (GSS), has also been introduced into *Prnp* by gene targeting ***(23)***.

3. Microinjection Transgenic Mice

The majority of transgenic studies of prion diseases have involved the incorporation of wild type or mutant PrP genes from different species into the genome of fertilized mouse embryos by DNA microinjection. Various chimeric gene constructs, incorporating PrP gene sequences from mouse and other species, have also been used to produce transgenic mice. The seminal transgenic experiments utilized cosmid clones containing PrP gene sequences isolated from Syrian hamster (SHa) and the I/lnJ strain of mice ***(24,25)*** and this approach was also used to produce transgenic mice expressing sheep PrP ***(26)***. The cos.Tet vector is a modification of the SHa cosmid vector and contains a 43-kb DNA fragment encompassing the PrP gene and approx 24 and 6 kb of 5' and 3' flanking sequences, respectively ***(27)***. The vector is designed to allow the convenient insertion of PrP coding sequences. A plasmid expression vector, based on the PrP gene derived from the I/lnJ PrP cosmid (phgPrP, or the "half-genomic" construct) has also been used to produce transgenic mice ***(28)***. A modified version of phgPrP, (MoPrP.Xho), has been produced with a unique XhoI site to allow the insertion of coding sequences downstream of exon 2 in Prnp ***(29)***. SHa and mouse PrP gene constructs, in which all intron sequences are removed (so-called "minigene constructs), fail to express PrP in the CNS, demonstrating the requirement for at least the smaller intron for efficient expression ***(24,28)***.

A transgenic expression vector (pMo53), has been engineered to incorporate the advantages and overcome the problems of the cosmid and plasmid

vectors ***(30)***. The vector contains 5' -flanking sequences derived from the I/lnJ PrP gene, which have been shown to directly express a chloramphenicol acetyl transferase reporter gene in mouse neuroblastoma Neuro-2A cells ***(31)***. The 1.2-kb 3'-flanking region consists of the polyadenylation signal from Prnp. The vector is designed to accept open reading frame cassettes at unique restriction sites immediately downstream from exon 2. The construct also allows the convenient replacement of the promoter region for ectopic expression studies.

4. Transgenic Models of Inherited Prion Diseases

Approximately 10–20% of human prion disease is inherited with an autosomal dominant mode of inheritance. More than 20 different missense and insertion mutations, which segregate with dominantly inherited neurodegenerative disorders, have been identified in the coding sequence of *PRNP*. Five of these mutations are genetically linked to loci controlling familial Creutzfeldt-Jakob disease (CJD), GSS, and fatal familial insomnia (FFI), which are all inherited human prion diseases that can be transmitted to experimental animals.

Additional compelling evidence for the protein-only hypothesis came from studies on transgenic mice that express a proline to leucine mutation at codon 101 of mouse PrP, equivalent to the human GSS P102L mutation. These mice (Tg[MoPrP-P101L]) spontaneously developed clinical and neuropathological symptoms similar to mouse scrapie at between 150 and 300 d of age ***(11,32)***. After crossing the mutant transgene onto the $Prnp^{0/0}$ background, the resulting Tg(MoPrP-P101L) $Prnp^{0/0}$ mice displayed a highly synchronous onset of illness at ~145 d of age, which shortened to ~85 d upon breeding to homozygosity for the transgene array. In addition, Tg(MoPrP-P101L) $Prnp^{0/0}$ mice had increased numbers of PrP plaques and more severe spongiform degeneration ***(11)***. In contrast, transgenic mice overexpressing wild-type mouse PrP at equivalent levels did not spontaneously develop neurodegenerative disease, although they had highly reduced mouse scrapie incubation times after inoculation with mouse prions. The serial propagation of infectivity from the brains of spontaneously sick Tg(MoPrP-P101L) mice to indicator Tg196 mice that express low levels of mutant protein, and do not otherwise develop spontaneous disease, demonstrated the production of infectious prions in the brains of these spontaneously sick mice ***(11,33)***. Prion infectivity from brain extracts of humans expressing the P102L GSS mutation was also propagated in transgenic mice expressing a chimeric mouse–human PrP gene with the P101L mutation ***(34)***. A synthetic peptide spanning residues 89–143, carrying the P101L mutation that was refolded into a β-sheet conformation, produced clinical signs of neurological dysfunction as well as neuropathological characteristics of prion disease, after ~360 d in 20/20 inoculated Tg196 mice. By contrast, Tg196 mice

receiving a substantially larger inoculum of the peptide in a non-β-sheet conformation exhibited no convincing evidence of experimental prion disease ***(35)***.

Unlike Tg(MoPrP-P101L) mice, gene-targeted mice, homozygous for the mouse PrP proline to leucine mutation at codon 101 did not spontaneously develop neurodegenerative disease ***(23)***. This result is perhaps not unexpected, since previous studies demonstrated that a threshold level of expression of P102L PrP was critical for the manifestation of spontaneous neurological disease in transgenic mice ***(11,32)***. Inoculation of gene-targeted MoPrP-P101L mice with prions from a patient with GSS, produced disease in 288 d. Disease was subsequently transmitted to wild-type mice at 226 d, and to gene targeted MoPrP-P101L mice after 148 d. Transmission of additional GSS cases will be important because previous studies suggested that the ability of some cases of GSS, but not others to transmit to wild-type mice, might be the result of strain effects ***(36)***.

Continued characterization of the various genetically programmed prion diseases in transgenic mice will provide the basis for studying the molecular mechanisms of phenotypic variability in these conditions. In contrast to Tg(MoPrP-P101L) mice, transgenic mice overexpressing a mutant mouse PrP gene with a glutamate to lysine mutation at codon 199, equivalent to the codon 200 mutation linked to familial CJD (E200K), did not spontaneously develop neurological disease ***(37)***. A mutation associated with GSS, in which the Tyrosine residue at codon 145 is mutated to a stop codon, was also modeled in transgenic mice. Expression of this truncated version of mouse PrP could not be detected in high copy number lines of these transgenic mice, referred to as Tg([MoPrP144#]), and neither uninoculated Tg(MoPrP144#) mice nor mice inoculated with mouse RML scrapie developed symptoms of neurodegenerative disease ***(38)***. Expression of a mouse PrP version of a nine-octapeptide insertion associated with prion dementia produced a slowly progressive neurological disorder in transgenic mice ***(39).*** At this stage it is not known whether infectious prions are produced in the brains of these mice.

Certain examples of prion disease, including transgenic models of GSS, occur without accumulation of protease-resistant PrP^{Sc} ***(11,32)***. Moreover, the time course of neurodegeneration is not equivalent to the time course of PrP^{Sc} accumulation in mice expressing low levels of PrP^{C} ***(7)***. Thus, it appears that accumulation of protease-resistant PrP^{Sc} may not be the sole cause of pathology in prion diseases. An alternative mechanism of PrP-induced neurodegeneration arose from transgenic studies of mutant forms of PrP that disrupt PrP biogenesis in the endoplasmic reticulum ***(40,41)***. Transgenic mice expressing mutations in the so-called "stop transfer effector region" between residues lysine 104–methionine 112 and the hydrophobic TM1 region between residues alanine 113–serine 135, spontaneously develop neurodegenerative dis-

ease and accumulate an aberrant form of PrP, termed "^{Ctm}PrP". Accumulation of ^{Ctm}PrP is also associated with a form of GSS in humans that segregates with the codon 117 mutation of *PRNP*. Accumulation of ^{Ctm}PrP as a cause of neurodegeneration is not exclusive to genetically programmed prion diseases. An elegant series of experiments in transgenic mice demonstrated that the effectiveness of PrP^{Sc} in causing neurodegeneration in transmissible prion diseases depends on the predilection of the host to accumulate ^{Ctm}PrP ***(41)***.

5. Transgenic Studies of Prion Species Barriers

The species barrier describes the difficulty with which prions from one species cause disease in another. In experimental studies, the initial passage of prions between species is associated with prolonged incubation times, with only a few animals developing illness. On subsequent passage in the same species, all animals become ill after greatly abbreviated incubation times. Prion species barriers have been eliminated by expressing PrP genes from other species or artificially engineered chimeric PrP genes in transgenic mice.

As a result of the species barrier, wild-type mice are normally resistant to infection with Syrian hamsters (SHa) prions. The seminal transgenic experiments by Scott et al. ***(24)*** that were designed to probe the molecular basis of the species barrier, demonstrated that expression of SHa PrP^{C} in transgenic (Tg[(SHaPrP]) mice, rendered them susceptible to SHa prions, and produced CNS pathology similar to that found in Syrian hamsters with prion disease. These studies were extended to show that the incubation period of SHa prions was inversely related to the level of expression of transgene-encoded PrP^{C} ***(42)***. Inoculation of Tg(SHaPrP) mice with mouse prions resulted in propagation of prions pathogenic for mice; inoculation with SHa prions resulted in the propagation of prions pathogenic for Syrian hamsters. These studies provided important clues about the mechanism of prion propagation involving association and conformational conversion of PrP^{C} into PrP^{Sc}, and suggested that, for optimum progression of the disease, the interacting species should be identical in primary structure. SHa PrP differs from mouse PrP at 16/254 amino acid residues ***(43,44)***. Chimeric SHa/mouse PrP transgenes produced prions with new properties. The MH2M transgene carries five amino acid substitutions found in SHa PrP lying between codons 94 and 188. Tg(MH2M) mice generated prions with an artificial host range, so that infectivity produced by inoculation with SHa prions could be passaged from Tg(MH2M) mice to wild-type mice, and infectivity produced by inoculation with mouse prions could be passaged from Tg(MH2M) mice to Syrian hamsters ***(45)***.

The infrequent transmission of human prion disease to rodents is also an example of the species barrier. Based on the results with Tg(SHaPrP) mice, it was expected that the species barrier to human prion propagation would be

abrogated in transgenic mice expressing human PrP. However, transmission of human prion disease was generally no more efficient in transgenic mice expressing high levels of transgene-expressed human PrP^C than in non-transgenic mice. In contrast, propagation of human prions was highly efficient in transgenic mice expressing a chimeric mouse–human PrP gene (Tg[MHu2M]) in which the region of the mouse gene between codons 94 and 188 was replaced with human PrP sequences ***(46)***. The barrier to CJD transmission in Tg(HuPrP) mice could be abolished by expressing HuPrP on a $Prnp^{0/0}$ background, demonstrating that mouse PrP^C inhibited the transmission of prions to transgenic mice expressing human PrP^C, but not to those expressing chimeric PrP ***(34)***.

To explain these and other data, it was proposed that the most likely mediator of this inhibition is an auxiliary non-PrP molecule, provisionally designated "protein X," which participates in the formation of prions by interacting with PrP^C to facilitate conversion to PrP^{Sc} ***(34)***. It has been proposed that protein X is bound to a form of PrP, referred to as "PrP^*," which exists in equilibrium with PrP^C ***(47)***. The PrP^*–protein X complex interacts with PrP^{Sc}, which induces a conformational change in PrP^*, the end result being two molecules of PrP with the infectious PrP^{Sc} conformation, which are free to induce conformational changes in additional PrP^* molecules during the infectious cycle. Although protein X has been postulated from genetic arguments, factors that interact with PrP^C, and are involved in its conversion to PrP^{Sc}, await identification and characterization. To date, no less than 12 proteins have been identified as potential PrP^C ligands (reviewed in *ref.* ***48***), but in no case has physiological relevance been confirmed.

In agreement with these experimental observations, the three-dimensional structure of recombinant PrP, derived from nuclear magnetic resonance spectroscopy, indicates two potential species–dependent recognition sites for protein–protein interactions on opposite molecular surfaces in the structured C-terminal region of PrP ***(49–51)***. A refinement of this model classified amino acid residues that differ between species according to their locations in the three-dimensional structure of PrP and the chemical properties of the amino acid residues ***(52)***. The region containing so-called "class A" residues is suggested as the binding site for protein X; the variable "class C" residues are predicted to be involved in interactions between PrP^C and PrP^{Sc}. A third region, consisting of "class B" residues, constitutes an internal hydrophobic core that may affect structural transformations following PrP^{Sc}–PrP^C interactions.

Based on the success of Tg(MHu2M) mice, transgenic mice expressing a chimeric mouse–bovine PrP construct (MBo2M) were produced. Both the MHu2M and MBo2M chimeras were constructed by exchanging homologous regions between codons 94 and 188 of human and mouse PrP and bovine and

mouse PrP coding sequences, using common restriction enzyme sites. However, although the nine amino acids from the human PrP coding sequence in this region of MHu2M are sufficient to confer susceptibility to CJD prions in transgenic mice, the eight amino acids from bovine PrP in this region of MBo2M are not sufficient to confer susceptibility to bovine songiform encephalopathy (BSE) prions, since Tg(MBo2M) mice did not develop disease after challenge with BSE ***(53)***. Transgenic mice expressing bovine PrP developed disease after inoculation with BSE, albeit with long incubation times between 250 and 300d ***(53)***.

Because of species-specific differences between mouse and bovine PrP, amino acid differences occur at residues 183 and 185 in α-helix 2 of MBo2M PrP, which are not present in MHu2M. The mouse and bovine PrP sequences also differ at position 202 in α-helix 3; the mouse and human sequences are equivalent in this region. All ungulate species that have succumbed to BSE infection encode valine at 183 and isoleucine at 202, raising the intriguing possibility that these residues may account for the differences in susceptibility of Tg(MHu2M) and Tg(MBo2M) mice to prion infection. The effects of exchanges at these positions on the capacity of transgenic mice to propagate BSE prions are currently being tested ***(30)***.

6. Transgenic Mice and Prion Strains

The degree of homology between PrP molecules in the host and inoculum is an important determinant of the species barrier, but an equally important component affecting prion transmission barriers is the strain of prion. Prion incubation times, profiles of neuropathological lesions in the CNS, and patterns of PrP^{Sc} deposition in the brain are features that have been used to characterize prion strains in inbred mice, hamsters, and transgenic mice ***(37,54–56)***. The importance of strain effects and species barriers is highlighted in the case of BSE, which has an unusually broad host range. As a result, BSE prions from cattle have caused disease in humans in the form of variant CJD (vCJD). The most convincing evidence that vCJD is the manifestation of BSE in humans has arisen from transmissions of vCJD prions to transgenic mice expressing bovine PrP, which produced incubation periods, neuropathology, PrP^{Sc} distribution, and PrP^{Sc} conformations that were identical to those produced by inoculation of BSE prions ***(57)***.

The passage of vCJD and BSE prions to inbred strains of mice has also been used to contend that prions causing BSE and vCJD are the same prion strain ***(55)***. These experiments are complicated by the transmission of prions across species barriers that prolong incubation times. A characteristic banding pattern of PrP^{Sc} glycoforms found in vCJD patients and BSE infected animals distinguishes vCJD PrP^{Sc} from the patterns observed in classical CJD ***(58,59)***. The

predominance of diglycosylated PrP^{Sc} in both BSE and vCJD brains has also been used as an argument for the two diseases being caused by the same prion strain ***(58,59)***. Transgenic mice expressing mutations at one or both glycosylation consensus sites have been studied to investigate the role of the Asn-linked oligosaccharides of PrP ***(60)***. Mutation of the first site altered PrP^{C} trafficking and prevented infection with two prion strains. Deletion of the second site did not alter PrP^{C} trafficking, but permitted infection with one prion strain and altered the pattern of PrP^{Sc} deposition.

Studies of different strains of transmissible mink encephalopathy, a prion disease of captive mink, suggested that different strains may be represented by different conformational states of PrP^{Sc} ***(61)***. Evidence supporting this concept emerged from transmission studies of inherited human prion diseases ***(37)***. Expression of mutant prion proteins in patients with FFI and familial CJD result in variations in PrP conformation reflected in altered proteinase K cleavage sites that generate PrP^{Sc} molecules with molecular weight of 19 kDa in FFI and 21 kDa in fCJD(E200K) ***(62)***. Extracts from the brains of FFI and fCJD(E200K) patients transmitted disease to Tg(MHu2M) mice after about 200 d on first passage, and induced formation of 19 and 21 kDa PrP^{Sc}, respectively ***(37)***. Upon second passage in Tg(MHu2M) mice, these characteristic molecular sizes remain constant, but the incubation times for FFI and fCJD prions diverge ***(63)***. These results indicate that PrP^{Sc} conformers function as templates in directing the formation of nascent PrP^{Sc} and provide a mechanism to explain strains of prions in which diversity is enciphered in the tertiary structure of PrP^{Sc}.

A sporadic form of fatal insomnia (SFI) has recently been described ***(56,64)***. Although patients with SFI have symptoms and neuropathological profiles indistinguishable from patients with FFI, they do not express the D178N mutant form of human PrP^{C}. SFI prions transmitted to Tg(MHu2M) mice were found to produce similar incubation periods and a pattern of neuropathology identical to transgenic mice infected with FFI prions ***(56)***. Analysis of PrP^{Sc} demonstrated equivalent conformations associated with SFI and FFI. These findings imply that the conformation of PrP^{Sc} not the amino acid sequence, determines the strain-specified disease phenotype.

7. Structure–Function Studies of PrP

The finding that the introduction of PrP transgenes into $Prnp^{0/0}$ mice restores susceptibility to scrapie opened the possibility for assessing whether a modified PrP^{C} molecule remains functional, at least insofar as it continues to be eligible for supporting prion propagation ***(65)***. Experiments in cell culture showed that deletion of the PrP sequence encoding residues that are removed from the N-terminus of PrP^{Sc} by limited proteolysis did not prevent the acqui-

sition of protease resistance and PrP^{Sc} formation ***(66)***. To further investigate the role of this region, a series of transgenic mice, expressing N-terminal deletions of varying extent, were produced. Deletions between codons 69 and 84, 32 and 80, 32 and 93 or 32 and 106 of the PrP coding sequence were able to restore susceptibility to scrapie in *Prnp*$^{0/0}$ mice ***(28,67)***, but deletions between codons 32 and 121 or 32 and 134 caused ataxia and degeneration of the granular layer of the cerebellum within 2–3 mo after birth ***(67)***. This defect was overcome by the co-expression of wild-type MoPrP, leading to the suggestion that truncated PrP may compete with a functionally similar non-PrP molecule for a common ligand.

A series of PrP coding sequence deletions, based on putative regions of secondary structure, were also expressed in transgenic *Prnp*$^{0/0}$ mice ***(38)***. These deletions were engineered in a modified PrP construct that lacks amino acid residues 23–88. Transgenic mice with additional deletions between codons 95 and 107, 108 and 121 and 141 and 176 remained healthy; transgenic mice with deletions at the C-terminus between codons 177 and 190 and 201 and 217, which disrupted the penultimate and last α-helix, showed neuronal cytoplasmic inclusions of PrP-derived deposits and spontaneously developed fatal CNS illnesses at 90–227 d of age.

Two of these deletion constructs were further characterized in transgenic mice, regarding their ability to support prion replication ***(68)***. Transgenic mice in which residues 23–88 were deleted remained resistant to infection, and this block to prion propagation was alleviated by further deleting residues between 141 and 176. In both cases, the block to prion propagation was overcome by co-expression of wild-type MoPrP.

8. Ectopic Expression Studies

Although the pathological consequences of prion infection occur in the CNS, PrP^{C} has a wide tissue distribution, and the exact cell types responsible for agent propagation and pathogenesis are still uncertain. PrP is expressed at highest levels in the CNS, but substantial amounts of PrP can be found in many tissues ***(69)***. Similarly, although the highest titers of infectious prions are found in the CNS, prions do accumulate in other organs, particularly in the spleen and other tissues of the reticuloendothelial system ***(70)***. In the CNS, PrP is expressed in neurons throughout the life of the animal, with levels of PrP mRNA varying among different types of neurons ***(44)***.

Transgenic mice expressing heterologous transgenes with cell-type-specific promoter/enhancer sequences linked to PrP coding sequences have been introduced into *Prnp*$^{0/0}$ mice in order to study the ability of specific cell types to support prion propagation. Transgenic mice in which the neuron-specific enolase promoter regulated SHa PrP expression indicated that neuron-specific

expression PrP^C was sufficient to mediate susceptibility to hamster scrapie after intracerebral inoculation ***(71)***. PrP is also normally expressed in astrocytes and oligodendrocytes throughout the brain of postnatal hamsters and rats ***(72)***. The level of glial PrP mRNA expression in neonatal animals is comparable to that of neurons, and increases twofold during postnatal development. Astrocytes have been found to be the earliest site of PrP^{Sc} accumulation in the brain ***(73)***, suggesting that these cells may play an important role in scrapie propagation and/or pathogenesis or that astrocytes themselves may be the cells in which prion replication occurs. Transgenic mice expressing SHa PrP under the control of the astrocyte-specific glial fibrilary acidic protein accumulated infectivity and PrP^{Sc} to high levels and developed disease after ~220 d ***(74)***.

The interferon regulatory factor-1 promoter/Eμ enhancer, lck promoter, and albumin promoter/enhancer have been used to direct PrP expression to the spleen, T lymphocytes and liver, respectively ***(75)***. High prion titers were found in the spleens of inoculated transgenic mice expressing PrP under the control of the interferon regulatory factor-1 promoter/Eμ enhancer, while mice expressing PrP under the control of the lck and albumin promoters failed to replicate prions. Finally, although previous reports found little or no prion infectivity in skeletal muscle, two types of transgenic mice, in which expression of PrP^C is directed exclusively to muscle under the control of the muscle creatine kinase and chicken α-actin promoters demonstrated that this tissue is capable of propagating prion infectivity ***(76)***. That muscle is competent to propagate prions raises the question of whether meat from prion disease infected animals may carry sufficient titers of prions to transmit disease to humans.

9. Conclusions and Future Prospects

While certain aspects of PrP^C to PrP^{Sc} conversion can be studied using in vitro systems, many ambiguities remain, and workers continue to rely heavily on in vivo analysis for studying prions and the prion diseases. Of the two general transgenic approaches, transgenesis by pronuclear microinjection has been the more informative for studying the biology of prion diseases. Although this approach results in lines with variable copy number and expression levels, these effects are easily controlled and offer a degree of flexibility that is not possible using gene replacement methods. In addition to providing insights into mechanisms of prion propagation, this approach has also resulted in rapid and sensitive infectivity assays for human and animal prions. Knockout approaches have yielded crucial information about the requirement of PrP expression for prion replication, but they have been less informative than expected in answering questions about the normal function of PrP. The recent discovery of *Prnd* offers exciting new approaches in this respect and the phenotypes of *Prnd* and double *Prnp/Prnd* knockouts are eagerly awaited.

Although transgenic studies demonstrated that species-specific amino acid differences influence the ability of prions from one species to cause disease in another species, studies in transgenic mice have also shown that strain diversity results from the ability of PrP^{Sc} to impart different tertiary structures to PrP^{C} by conformational templating. A major goal of future studies will be to determine the interplay between PrP primary structure and conformation in determining prion transmission barriers. In light of BSE transmission to humans, understanding the risk of prion infections from other sources is clearly of paramount importance. Transgenic models of other naturally occurring prion diseases, such as chronic wasting disease of deer and elk, are required to better understand modes of transmission and the pathogenesis of disease, as well as to improve diagnosis and control of these diseases. In addition, the complex genetics of scrapie susceptibility in sheep presents many opportunities for transgenic investigations.

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8

Prions: From Neurografts to Neuroinvasion

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1. Introduction

The prion hypothesis states that the partially protease-resistant and detergent-insoluble prion protein (PrP^{Sc}) is identical with the infectious agent, and lacks any detectable nucleic acids. Since the latter discovery, transgenic mice have contributed many important insights to the field of prion biology. The prion protein (PrP^{C}) is encoded by the *Prnp* gene, and disruption of *Prnp* leads to resistance to infection by prions. Ectopic expression of PrP^{C} in PrP^{C}-knockout mice proved a useful tool for the identification of host cells competent for prion replication. Finally, the availability of PrP^{C}-knockout mice, and transgenic mice overexpressing PrP^{C}, allowed selective reconstitution experiments aimed at expressing PrP^{C} in neurografts or in specific populations of hemato- and lymphopoietic cells. The latter studies helped elucidate some of the mechanisms of prion spread and disease pathogenesis.

2. PrP-Knockout Mice and Their Phenotypes

If the protein only hypothesis is correct, PrP^{C} functions as a substrate for the PrP^{Sc}-mediated conversion of PrP^{C} into new PrP^{Sc} molecules. As a consequence of this hypothesis, an organism lacking PrP^{C} should be resistant to scrapie and unable to propagate the infectious agent. The mice generated by Büeler et al. ***(1)*** carry a targeted disruption of the *Prnp* gene. This was achieved by homologous recombination in embryonic stem cells. In the disrupted *Prnp* allele, 184 codons of the Prnp coding region (which consists of 254 codons) were replaced by a drug-resistance gene as selectable marker. A second line of PrP^{C} knockout mice was generated by Manson et al. ***(2)*** by inserting a select-

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able marker into the PrP^C open reading frame, which leads to a disruption of the coding region of Prnp. Sakaguchi et al. created a third PrP^C-knockout line, in which the whole PrP^C open reading frame and about 250 bp of the 5' intron and 452 bp of 3' untranslated sequences were replaced with a drug-resistance gene ***(3)***. Although both the Büeler and Sakaguchi mice were on a mixed genetic (129/Sv × C57BL) background, the mice generated by Manson were bred on a pure 129/Ola background. According to the terminology that has become customary in the literature, and to which we abide in this manuscript, the Büeler mice have been designated $Prnp^{0/0}$ while the Manson and the Sakaguchi mice are termed $Prnp^{-/-}$.

The phenotype of the Prnp-knockout mice was of great interest, because it was proposed that PrP^C, which is a ubiquitously expressed neuronal protein, may have a housekeeping function ***(4)***.Yet the homozygous PrP^C-knockout mice generated by Büeler et al. and Manson et al. were viable and showed no overt phenotypic abnormalities, suggesting that PrP^C does not play a crucial role in development or function of the nervous system ***(1,2)***. The $Prnp^{0/0}$ mice show no behavioral impairment and perform like the wild type controls in spatial learning tests ***(5)***. Detailed analysis revealed electrophysiological defects, such as weakened γ-aminobutyric acid type A receptor-mediated fast inhibition and impaired long-term potentiation in the hippocampus for the Zurich and Edinburgh PrP^C-knockout mice compared to their corresponding wild-type counterparts, indicating that PrP^C may play a role in synaptic plasticity ***(6)***. Tobler et al. ***(7)*** reported altered sleep patterns and rhythms of circadian activity in the Büeler and Manson mice.

The PrP^C-null mice derived by Sakaguchi et al. developed severe progressive ataxia starting from 70 weeks of age ***(3)***. Analysis of the brains of affected animals revealed extensive loss of cerebellar Purkinje cells ***(8)***. Because no such phenotype was observed in the other two lines of PrP^C-knockout mice, it seems likely that this phenotype is not the result of the lack of PrP^C, but rather results from deletion of flanking sequences. A Purkinje cell-specific enhancer was proposed ***(9)*** to be contained within the second intron of Prnp. The report ***(10)*** that expression of a *Prnp* transgene can rescue this phenotype argues against the hypothesis that the phenotype was caused by deletion of a regulatory element, rather than of the Prnp reading frame ***(10)***. Recently, evidence has been forthcoming that the phenotype observed in the Sakaguchi mice is caused by upregulation of a second *Prnp*-like gene located 16 kb downstream for the *Prnp* gene ***(11)***. The exact mechanism of this process is still under discussion ***(12)***.

3. PrP-Null Mice Are Resistant to Scrapie

One of the milestones in scrapie research was the inoculation of PrP^C null mice with mouse adapted scrapie strains. All three PrP^C null mouse lines were

resistant to scrapie. The $Prnp^{0/0}$ mice generated by Büeler et al., inoculated with the RML isolate of mouse-adapted prions, remained healthy for their whole life-span, and did not show any signs of scrapie-typical neuropathology ***(13)***. This observation was confirmed using different PrP^C-null mice with different mouse-adapted scrapie inocula ***(3,14)***. Mice hemizygous for the disrupted Prnp gene ($Prnp^{0/+}$) showed partial resistance to scrapie infection, as manifested by prolonged incubation times of ~290 d as compared to ~160 d in the case of $Prnp^{+/+}$ mice. There is a strict correlation between the levels of PrP^C in the host and incubation times until terminal disease; the severity of the disease, in terms of neuropathological changes in the brain and levels of prion infectivity, were not dependent on the PrP^C level ***(14,15)***. All of the experiments show that the amount of PrP^C present in the brain seems to be the rate-limiting step in the development of the disease. Therefore, therapeutic efforts aimed to reduce the amount of PrP^C may be effective.

4. Structural Implications of the Infectivity of PrP

Limited proteolysis of PrP^{Sc} cleaves off the N-terminus and a fragment termed $PrP^{27\text{-}30}$ remains. This portion of PrP^{Sc} is still infectious, meaning that the last 60 amino-proximal residues of PrP^{Sc} are not required for infectivity ***(16,17)***. PrP^C lacking residues 23–88 can be converted into protease-resistant PrP^C in scrapie-infected neuroblastoma cells ***(18)***. An important question arising from these experiments is whether N-terminally truncated PrP^C molecules can support prion replication in mice. In order to address this question transgenic mice, expressing N-terminal deletions of the prion protein on a PrP^C null background, were established. These mutant PrP^C mice with amino-proximal deletions of residues 32–80 and 32–93, corresponding to truncations of 49 and 63 residues, restore scrapie susceptibility, prion replication, and formation of truncated PrP^{Sc} in PrP^C deficient mice ***(19)***.

The data obtained from these experiments demonstrate that the octapeptide region encompassing residues 51–90 of murine PrP^c is dispensable for scrapie pathogenesis. This is remarkable, because additional octapeptide repeats instead of the normal five segregate with affected individuals in families with inherited Creutzfeldt-Jakob disease ***(20)***, and because expression of a mutant PrP^c with a pathological number of octarepeats induces a neurodegenerative disease in transgenic mice ***(21)***.

5. Mice Expressing Truncated PrP^c Show Severe Ataxia

Nuclear magnetic resonance studies helped to reveal the three–dimensional structure of PrP^C. Full-length, mature PrP^C seems to have a highly flexible N- terminal tail that lacks ordered secondary structures extending from residue 23 to 121; the C-terminal part of PrP^C consists of a stably folded globular

domain ***(22,23)***. The highly flexible tail, part of which is protease-sensitive in PrP^{Sc}, comprises the most conserved region of PrP^{C} across all species examined ***(24)***. Following these structural studies, the possibility that the flexible tail may play a role in the conformational transition of PrP^{C} to PrP^{Sc} was proposed ***(22,25)***. To further analyze the importance of the flexible tail in regard to scrapie susceptibility, Shmerling et al. ***(19)*** generated amino-proximal deletions of residues 32–121 and 32–134, and expressed them as transgenes in PrP^{C}-deficient mice. Mice overexpressing these transgenes developed severe ataxia and neuronal death limited to the granular layer of the cerebellum, as early as 1–3 mo of age. No pathological phenotype was observed in transgenic mice with shorter deletions encompassing residues 32–80, 32–93 and 32–106. Because of the selective degeneration of granule cells in the cerebellum, a nonspecific toxic effect elicited by the truncated PrP^{C} can be ruled out. Another argument for a specific effect is the fact that neurons in the cortex, and elsewhere, express truncated PrP^{C} at similar levels, but do not undergo cell death by apoptosis. One copy of a wild-type Prnp allele, introduced into these mice, completely abolishes the phenotype. Based on these results, a model was proposed in which truncated PrP^{C} acts as dominant negative inhibitor of a functional homolog of PrP^{C}, with both competing for the same putative PrP^{C} ligand ***(19)***.

A different spontaneous neurologic phenotype was reported in mice carrying PrP^{C} transgenes with internal deletions corresponding to either of the two carboxy-proximal α-helices. Two transgenic mouse lines generated on the $Prnp^{0/0}$ background, expressing mutant PrP^{C} with deletions of residues 23–88 and either residues 177–200 or 201–217, developed central nervous system (CNS) dysfunction and neuropathological changes characteristic of a neuronal storage disease ***(26)***. Because deletion of residues 23–88 alone did not lead to a spontaneous phenotype, it was concluded that ablation of either of the two C-terminal α-helices is sufficient to cause this novel CNS illness. Ultrastructural studies indicated extensive proliferation of the endoplasmic reticulum, and revealed accumulation of mutant PrP^{C} within cytoplasmic inclusions in enlarged neurons.

A completely new light is shed on all of these studies by the discovery of a Prnp like gene, named Prnd that encodes for a protein named Doppel, (German for double) (Dpl), located 16 kb downstream of the murine *Prnp* gene ***(11)***. Elevated levels of Prnd RNA are present in certain strains of PrP^{C} knockout mice that develop neurological symptoms. It was shown that mice with a truncated Prnp transgene, lacking the N-terminus, and therefore devoid of the conserved 106–126 amino acid region, develop granule cell degeneration. This phenotype can be rescued by introduction of single intact PrP^{C} allele. The information from the PrP^{C} knockout mice that overexpress Dpl and the data from the truncated Prnp transgenic mice, led to the hypothesis that PrP^{C} inter-

acts with a ligand to produce an essential signal. In PrP^C knockout mice, a PrP^C-like molecule, with a lower binding affinity, could substitute for PrP^C. In the N-terminated transgenic mice, the truncated PrP^C could bind the ligand with high affinity, without eliciting the survival signal. Dpl could act in a similar way, and produce its effects through a competition with PrP^C for the PrP^C ligand, thus blocking an important signal ***(11,12)***. Knockout mice for the Prnd gene should help to clarify whether this proposed model is accurate.

6. Neurografts in Prion Research

$Prnp^{0/0}$ mice show normal development and behavior ***(1,2)***, which has led to the hypothesis that scrapie pathology is caused by PrP^{Sc} deposition, rather than by depletion of cellular PrP^C ***(6)***. This was confirmed by cell culture experiments, which showed that a part of PrP^C, corresponding to amino acids 106–126, acts as a neurotoxin. The peptide corresponding to residues 106–126 of PrP^C has a high intrinsic ability to polymerize into amyloid-like fibrils ***(27)***. If the depletion of cellular PrP^C is really the reason for scrapie pathology, lack of PrP^C may result in embryonic or perinatal lethality, especially since PrP^C is encoded by a unique gene, for which no related family members have been found. Until now, there is no stringent mouse model in which PrP^C can be depleted in an acute fashion. In this case, the depletion of PrP^C may be much more deleterious than its lack throughout development, because the organism may then not have the time to enable compensatory mechanisms.

The neurografting technique offers an attractive model to study the question of neurotoxicity of PrP^{Sc}. By grafting, one can expose brain tissue of $Prnp^{0/0}$ mice to a continuous source of PrP^{Sc}. Mid-gestation neuroectoderm, overexpressing PrP^C, was grafted into the brain of PrP^C-deficient mice, using well-established protocols ***(28,29)***. Following intracerebral inoculation with scrapie prions, neuroectodermal grafts accumulated high levels of PrP^{Sc} and infectivity, and developed severe histopathological changes characteristic of scrapie. It was shown that, at later time-points, substantial amounts of graft-derived PrP^{Sc} migrated into the host brain, and, even in areas distant from the grafts, substantial amounts of infectivity were detected ***(9,30)***. Nonetheless, even 16 mo after transplantation and infection with prions, no pathological changes were detected in the PrP^C-deficient tissue, not even in the immediate vicinity of the grafts or the PrP^{Sc} deposits. These results suggest that PrP^{Sc} is inherently nontoxic, and that PrP^{Sc} plaques found in spongiform encephalopathies may be an epiphenomenon, rather than a cause of neuronal damage ***(31)***. Maybe the PrP^{Sc}-containing plaques must be formed and localized intracellularly in order to be neurotoxic. If this is the case, plaques that are localized extracellularly may not be toxic. This would explain the absence of pathological changes outside the PrP^C-containing grafts.

Because the host mice harboring a chronically scrapie-infected neural graft did not develop any signs of disease, they enabled us not only to study the effects of prions on the surrounding tissue, but were also an ideal model to assess changes occurring during the progression of scrapie disease in neuronal tissue. The possibility of studying late time-points, after infection with the scrapie agent, was useful in order to observe phenomena that cannot be seen in PrP^C-containing mice, because these mice develop clinical symptoms, eventually leading to earlier death. With increasing incubation time, grafts underwent progressive astrogliosis and spongiosis, which were accompanied by loss of neuronal processes within the grafts and subsequent destruction of the neuropil (**Fig. 1**). The latest studied time-point was 435 d after inoculation; grafts showed an increase of cellular density probably caused by astroglial proliferation and a complete loss of neurons. Magnetic resonance imaging in vivo, using gadolinium as a contrast enhancing medium, revealed a progressive disruption of the blood–brain barrier in scrapie-infected grafts during the course of the disease ***(32)***. These findings confirmed several predictions about the pathogenesis of spongiform encephalopathies, primarily that scrapie leads to selective neuronal loss, and that astrocytes and perhaps other neuroectodermal cells, while being affected by the disease, can survive and maintain their phenotypic characteristics for long periods of time.

In other experimental models such as experimental hamster scrapie, disruption of the blood-brain barrier was also visible ***(33)*** yet no such observations were made in human spongiform encephalopathies. The localized blood-brain barrier disruption in chronically infected grafts may contribute to the spread of prions from grafts to the surrounding brain, as described previously ***(30)***. It may also account for the pattern of accumulation of protease-resistant PrP^C within the white matter, and in brain areas surrounding the grafts. The accumulation of PrP^{Sc} in nonaffected neuropil surrounding the graft could also be explained through vasogenic diffusion from the affected graft toward the host brain.

7. Mechanism of Prion Spread in the Central Nervous System

Transmission of prion diseases can be accomplished by injecting infected brain homogenate into suitable recipients. It has been shown that infection is possible by a number of different inoculation routes. Intracerebral inoculation is the most effective method for transmission of spongiform encephalopathies and may even facilitate circumvention of the species barrier. Other modes of transmission are oral uptake of the agent ***(34–36)*** intravenous and intraperitoneal injection ***(37)*** as well as conjunctival instillation ***(38)***, implantation of corneal grafts ***(39)***, and intraocular injection ***(40)***. Intraocular injection is an elegant way of studying the neural spread of the agent, because the retina is a part of the (CNS), and intraocular injection does not produce direct physical

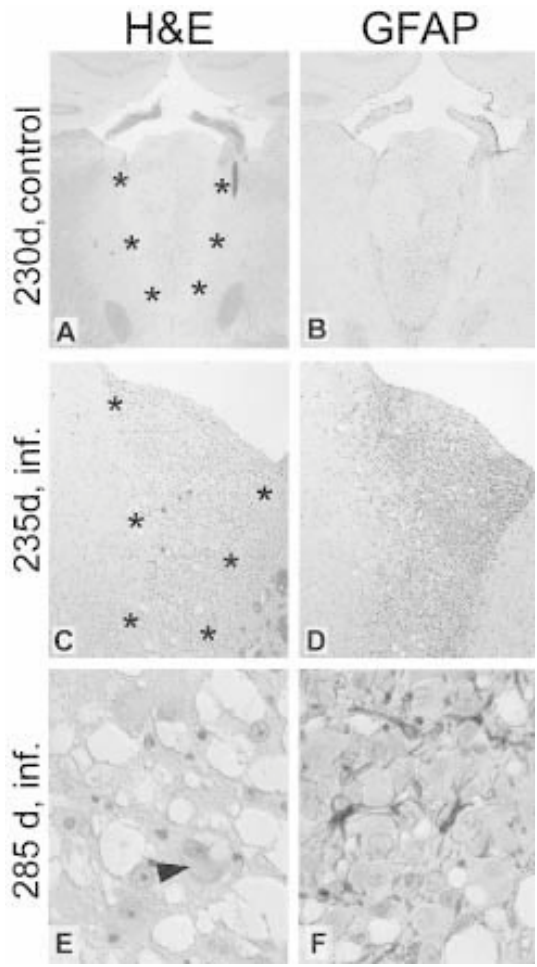

Fig. 1. Noninfected and scrapie-infected neural grafts in brains of $Prnp^{0/0}$ mice. Upper row (**A,B**) Healthy control graft 230 d after mock inoculation. The graft is located in the third ventricle of the recipient mouse (**A**, *see* asterisks, hematoxylin and eosin), and shows no spongiform change, little gliosis (**B**, immunostain for glial fibrillary acidic protein [GFAP]) (**C,D**) Scrapie-infected graft 235 d after inoculation with increased cellularity (**C**), brisk gliosis (**D**). Bottom row: High magnification of a similar graft shows characteristic pathological changes in a chronically infected graft. (**E**) Appearance of large vacuoles and ballooned neurons (arrow). In the GFAP immunostain (**F**), astrocytes appear wrapped around densely packed neurons.

trauma to the brain, which may disrupt the blood-brain barrier and impair other aspects of brain physiology. The assumption that the spread of prions within the CNS occurs axonally rests on experimental data gathered from mainly mice

intraocularly. Fraser ***(40)*** could show that the sequential development of spongiform changes follows the retinal pathway in a fashion that suggests transport along axons or in axons.

It has been repeatedly shown that expression of PrP^C is required for prion replication ***(13,41)*** and for neurodegenerative changes to occur ***(30)***. To investigate whether spread of prions within the CNS is dependent on PrP^C expression in the visual pathway, PrP^C-producing neural grafts were used as sensitive indicators of the presence of prion infectivity in the brain of an otherwise PrP^C-deficient host.

Following inoculation with prions into the eye of grafted $Prnp^{0/0}$ mice, none of the grafts showed signs of spongiosis, gliosis, synaptic loss, or PrP^{Sc} deposition. In one instance, the graft of an intraocularly inoculated mouse was assayed and found to be devoid of infectivity. Therefore, it was concluded that infectivity administered to the eye of PrP^C-deficient hosts cannot induce scrapie in a PrP^C-expressing brain graft ***(42)***.

One problem encountered while conducting work with PrP^C-containing grafts in $Prnp^{0/0}$ mice is that PrP^C-producing tissue may induce an immune response to PrP^C ***(43)***, this in turn could lead to neutralization of infectivity. Indeed, analysis of sera from grafted mice revealed significant anti-PrP^C antibody titers ***(42)***. It was shown that PrP^C, presented by the intracerebral graft (rather than the inoculum or graft-borne PrP^{Sc}), was the offending antigen. In order to definitively rule out the possibility that prion transport was disabled by a neutralizing immune response, these experiments were repeated in mice tolerant to PrP^C, namely the $Prnp^{0/0}$ mice transgenic for the PrP^C coding sequence under the control of the lck-promoter. These mice overexpress PrP^C on T-lymphocytes, but were resistant to scrapie and did not replicate prions in brain, spleen, and thymus after intraperitoneal inoculation with scrapie prions ***(44)***. Upon grafting with PrP^C-overexpressing neuroectoderm, these mice do not develop antibodies to PrP^C, presumably because of clonal deletion of PrP^C immunoreactive lymphocytes. The results obtained from the previous experiments were confirmed. Intraocular inoculation with prions did not provoke scrapie in the graft, supporting the conclusion that lack of PrP^C, rather than immune response to PrP^C, prevented prion spread ***(42)***. Therefore, PrP^C appears to be necessary for the spread of prions along the retinal projections and within the CNS.

The conclusion that can be drawn from these results is that intracerebral spread of prions is based on a PrP^C -paved chain of cells, perhaps because they are capable of supporting prion replication. When such a chain is interrupted by interposed cells that lack PrP^C, as in the case described here, the transport of infectivity to the target tissue is impaired. One possible explanation for this is that prions require PrP^C for propagation across synapses: PrP^C is present in the

synaptic region ***(45)***, and certain synaptic properties are altered in $Prnp^{0/0}$ mice ***(6,46)***. Another possibility is that transport of prions within (or on the surface of) neuronal processes occurs in a PrP^{C}-dependent fashion, through conversion of PrP^{C} by adjacent PrP^{Sc}. In this mode of transport, infectivity moves along PrP^{C}-expressing tissue *per continuitatem* in a domino-stone-like manner ***(47)***.

8. Mechanisms of Prion Spread from Extracerebral Sites to the Central Nervous Sytem

Even though intracerebral inoculation of prions is the most efficient way of transmitting prion diseases, from an epidemiological point of view oral uptake of prions may be more relevant than intracerebral transmission, because this way of prion uptake is thought to be responsible for the bovine spongiform encephalopathy epidemic and its transmission to a variety of species, including humans ***(48,49)***. Prions can find their way through the body to the brain of their host, yet histopathological changes have not been identified in organs other than the CNS. Upon extracerebral infection with prions, a constant feature is the long incubation time until the development of clinical disease, which may be explained by multiplication of prions in reservoirs. One possible candidate for such a reservoir is the lymphoreticular system (LRS). This is supported by the finding that prion replication in lymphoid organs always precedes prion replication in the CNS, even if infectivity is administered intracerebrally (**Fig. 2**; ***50***). Prions may multiply silently in reservoirs during the incubation time of the disease. Infectivity can accumulate in all components of the LRS, including lymph nodes and intestinal Peyer's patches, where prions replicate almost immediately after oral administration of prions to mice ***(51)***. Recently, it was shown that variant Creutzfeldt Jakob disease prions accumulate in the lymphoid tissue of tonsils in such large amounts that PrP^{Sc} can easily be detected with antibodies on histological sections ***(52)***.

A wealth of early studies point to the importance of prion replication in lymphoid organs yet little is known about which cells support prion propagation in the LRS. Whole-body ionizing radiation studies in mice ***(53)***, after intraperitoneal infection, have suggested that the critical cells are long-lived. The follicular dendritic cell (FDC) would be a prime candidate, and indeed PrP^{Sc} accumulates in such cells of wild type and nude mice (which have a selective T-cell defect) ***(54)***. In addition, when mice with severe combined immunodeficiency (SCID), whose FDCs are thought to be functionally impaired, are challenged with the scrapie agent intraperitoneally, they do not develop the disease, nor is there any replication of prions in the spleen ***(55)***. Further support that the FDC are essential for the replication of prions in the LRS came from a study in which chimeric mice, with a mismatch in the PrP^{C} status between FDC and other cells of the immune system, were generated.

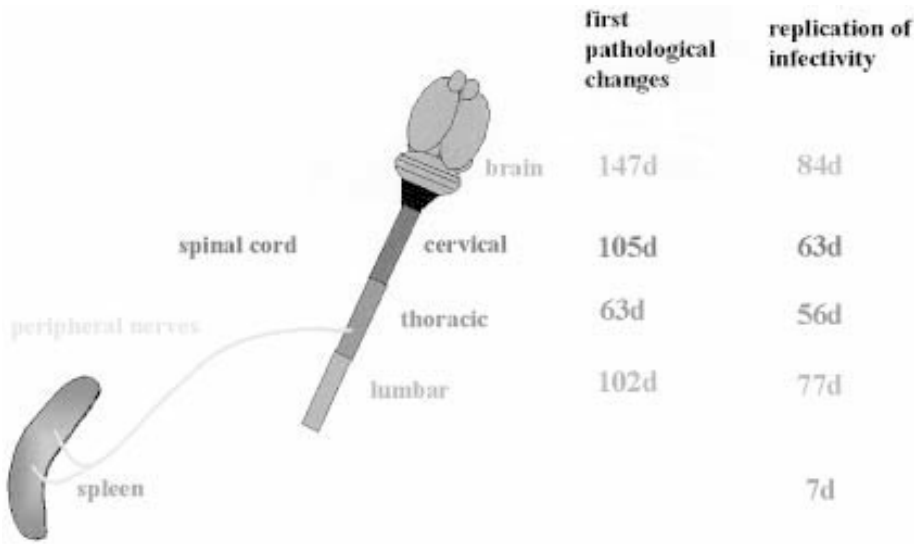

Fig. 2. Detection of PrP^{Sc} or detection of pathological changes in the spleen, the spinal cord (first number) and detection of replication of the infectious agent (second number), in the spleen, the spinal cord, and the CNS of mice, following intraperitoneal administration of the infectious agent. Infectivity and PrP^{Sc} can be detected in the LRS at early time-points. The first pathological changes and detection of infectivity within the CNS can be observed in the thoracic spinal cord.

That study demonstrated that replication of prions in the spleen depends on PrP^{C}-expressing FDCs ***(56)***. Upon reconstitution of SCID mice with wild-type spleen cells susceptibility to scrapie is restored after peripheral infection ***(57)***. These findings suggest that components of the immune system are required for efficient transfer of prions from the site of peripheral infection to the CNS.

To study the role of the immune system in more detail, we used a panel of immune-deficient mice that were inoculated intraperitoneally with prions. Defects in the T-cell lineage had no apparent effect, but all mutations that disrupted the differentiation of B-cells prevented the development of clinical scrapie ***(58)***. From these results, one can conclude that B-cells are important for the development of scrapie, after peripheral infection. Do B-cells physically transport prions all the way from the periphery to the CNS? This possibility seems unlikely, since lymphocytes do not normally cross the blood-brain barrier unless they have a specific reason to do so (e.g., during an inflammatory reaction). Furthermore, PrP^{Sc} could be demonstrated in up to 30% of B-cell-deficient mice without any signs of clinical disease ***(59)***. How is the spread of prions accomplished within the body? Perhaps, prions administered

to peripheral sites are first brought to lymphatic organs by mobile immune cells, such as B-cells. Once infection has been established in the LRS, prions invade peripheral nerve endings and find access to the CNS ***(60,61)***.

9. Role of B-Lymphocytes in Neuroinvasion

The replication of prions ***(13)*** and their transport from the periphery to the CNS ***(62)*** are dependent on expression of PrP^C. With respect to the results described in the previous paragraph, we examined whether expression of PrP^C by B-cells was necessary to support neuroinvasion. In order to study this matter, the LRSs of mice with various immune defects were repopulated by adoptive transfer of hematopoietic stem cells that expressed or lacked expression of PrP^c.

Adoptive transfer of either $Prnp^{+/+}$ or $Prnp^{0/0}$ fetal liver cells (FLCs) induced formation of germinal centers in spleens of recipient mice and differentiation of FDCs, as visualized by staining with antibody FDC-M1 ***(63)***. However, no FDCs were found in B- and T-cell deficient mice reconstituted with FLCs from *μMT* embryos (B-cell-deficient), consistent with the notion that B-cells, or products thereof, are required for FDC maturation.

Mice reconstituted in the fashion explained above were challenged intraperitoneally with scrapie prions. All mice that received FLCs of either genotype, $Prnp^{+/+}$ or $Prnp^{0/0}$, from immunocompetent donors, succumbed to scrapie after inoculation with a high dose of prions, and most mice after a low dose. Susceptibility to disease could not be restored upon transfer of FLCs from *μMT* donors; omission of the adoptive transfer procedure, did not restore susceptibility to disease in any of the immune-deficient mice challenged with the low dose of prions. With the high-dose inoculum, susceptibility to scrapie could be restored, even in the absence of B-cells and FDCs. B and T cell deficient mice reconstituted with bone marrow from mice that lack T cells except those expressing TCRα/ but have intact B cells — regained susceptibility to scrapie, again confirming the dependency of infectibility on the presence of B-cells. When individual samples of brain and spleen from the scrapie-inoculated bone marrow chimeras were transmitted into highly susceptible indicator mice, we observed restoration of infectious titers and PrP^{Sc} deposition in spleens and brains of recipient mice either carrying $Prnp^{+/+}$ or $Prnp^{0/0}$ donor cells ***(63)***.

B-cells are clearly a cofactor in peripheral prion pathogenesis, but the identity of those cells in which prions actually replicate within lymphatic organs is uncertain. In a further step to clarify this issue, we investigated whether splenic PrP^{Sc} was associated with FDCs in repopulated mice. Double-color immunofluorescence confocal microscopy revealed deposits of PrP^c-immunoreactive material in germinal centers, which appeared mostly colocalized with the follicular dendritic network in spleens of reconstituted mice.

Taken together, all the information we have gained with the above-described experiments support the hypothesis that cells whose maturation depends on B-cells are responsible for accumulation of prions in lymphoid tissue, such as the spleen. FDCs, although their origin remains obscure, are a likely candidate for the site of prion replication, because their maturation correlates with the presence of B-cells and their products.

10. Role of the Peripheral Nervous System in Prion Neuroinvasion

The question of whether accumulation of prions in the LRS is necessary or not, in order to obtain neuroinvasion, is still under discussion. There is substantial evidence for both lines of argumentation. Splenectomy prolongs the incubation time, and the key role of the LRS, including the FDC, in neuroinvasion clearly speaks in favor of an essential role of the LRS in neuroinvasion ***(56,58,60)***. On the other hand, several studies have shown that neuroinvasion can be achieved in mice devoid of an intact immune system, or in mice without PrP^C expression on cells of the LRS ***(57,64)***. One argument, which speaks in favor of the essential role of the LRS in neuroinvasion, is that this system could represent the reservoir responsible for the long incubation times until the onset of clinical disease. Yet, it is also conceivable that the reservoir could be constituted by a part of the peripheral nervous system (PNS). It was shown that PrP^{Sc} is detectable in enteric ganglia after oral infection of hamsters with the scrapie agent ***(65)***. In a different experimental setup, PrP^{Sc} was detectable after intraperitoneal infection of hamsters and sheep in ganglia belonging to the autonomous nervous system, such as enteric and dorsal root ganglia ***(66)***.

One important question is the function of PrP^C in the process of neuroinvasion. Indirect evidence points to a crucial role for PrP^C expression on the PNS in neuroinvasion via the PNS. PrP^C-expressing neurografts in $Prnp^{0/0}$ mice do not develop scrapie histopathology after intraperitoneal or intravenous iv inoculation with prions, and no infectivity is detectable in spleens. Following reconstitution of the host lymphohemopoietic system with PrP^C-expressing cells, prion titers in the spleen are restored to wild-type levels but PrP^C-expressing grafts fail to develop scrapie upon intraperitoneal or intra venous infection with prions (**Fig. 3**; ***62***).

In order to study the role of the PNS, and especially the function of PrP^C expression on the PNS, we have developed a method to express genes of interest in the PNS. This system is used to express PrP^C selectively in the sciatic nerve of a PrP^C knockout mouse ***(67)***. With this system, one should be able to answer some of the open questions concerning neuroinvasion and the role of PrP^C expression in the PNS.

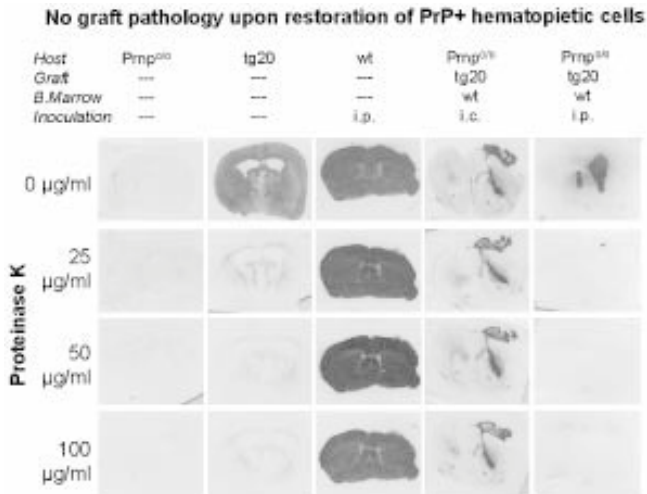

Fig. 3. Accumulation of PrP^{Sc} in brain grafts. Histoblots showing immunoactive PrP^{c} in brain sections natively (first row) and after digestion with increasing levels of proteinase K (PK) (second through fourth row). *Prnp*^{0} mice (first column) show no immunoreactivity; mock-inoculated tg20 mice (which overexpress PrP^{C}) show PK-sensitive PrP^{C} (second column), but no PK-resistant PrP^{Sc}. Terminally sick scrapie-nfected wilde-type mice contain large amounts of both PrP^{C} and PrP^{Sc} (third column). *Prnp%*, whose bone marrow has been reconstituted with wild-type FLCs, accumulate PrP^{Sc} in their PrP^{C}, overexpressing grafts after ic (fourth column), but not after ip prion administration (fifth column).

Even if the accumulation of prions within the LRS is essential to achieve neuroinvasion, one common pathway for neuroinvasion could be via the PNS. How the transfer of infectivity from cells belonging to the LRS to peripheral nerves is accomplished is still a matter of discussion. Access to peripheral nerves is facilitated if myelination of the nerves is reduced or absent ***(68)***. Therefore, the mantle zone of lymph follicles, which are innervated by terminal unmyelinated nerve fibers, could be the entry point of the scrapie agent into the PNS. Possibly, this is a region where processes belonging to FDC could be in close contact with nerve fibers. Once invasion of the PNS has taken place, the agent probably travels along the peripheral nerves to the CNS. The exact mode of transport within the PNS remains to be discovered: Axonal and nonaxonal modes of transport are conceivable: For PrP^{C}, transport in the fast axonal pathway was shown ***(69)***; for PrP^{Sc}, the mode of transport has only been studied in an indirect fashion, by comparing the incubation times of mice inoculated intraneurally to mice that were inoculated extraneurally or intracerebrally. In the case of intraneural injection of the agent, transport of the

scrapie agent to the CNS occurred faster than in extraneurally injected mice. The actual rate of spread within the PNS was calculated to be around 1–2 mm/d ***(68)***. Obviously, this rate of spread does not correspond to the fast axonal transport. Recently, data was presented showing that PrP^{Sc} localizes adaxonally within the PNS of intraperitoneally infected hamsters and sheep: this speaks in favor of a nonaxonal transport mechanism within the PNS ***(66)***. Considering the advances in visualization of axonal and nonaxonal transport mechanisms, clarification of the exact transport mechanism of PrP^{Sc} should be possible in the near future.

Based on the assumption that prions are transported in the PNS, an important question relates to the anatomical identity of the nerves in which transport occurs. In the case of direct intraneural injection of prions, transport of infectivity to the CNS is accomplished via the injected nerve. Following intraperitoneal or oral infection, prions accumulate in lymphatic organs, which are predominantly innervated by nerve fibers belonging to the sympathetic nervous system (SNS). The first hints that the scrapie agent may invade the CNS using nerve fibers of the SNS came from studies aimed at unraveling the dynamics of vacuolation and replication in the CNS. Following peripheral inoculation of the scrapie agent, the first pathological changes, such as spongiosis, as well as replication of the infectious agent, appear in the midthoracic spinal cord, in the same level where nerves from the SNS enter the spinal cord (**Fig. 2**; ***70,71).*** More recent studies describe an additional access route that by passes the spinal cord, using nerves of the parasympathetic nervous system, namely, the vagal nerve. This alternative route seems to be highly significant when animals are challenged via the oral route of infection ***(72,73)***.

Conclusion

Peripheral pathogenesis of prion diseases is here defined as the process starting with the contact of the infectious agent with extracerebral sites, and eventually resulting in brain disease. This process occurs in distinct sequential phases. The earliest event in disease progression is certainly accumulation of prions in the LRS. This process is dependent on components of the host immune system. Whether prions replicate, or merely accumulate in the LRS is not known with certainty. FDC play a major role in this process, but the details are still under discussion. In order to achieve efficient neuroinvasion, either B-cells *per se*, or their products, are essential. One B-cell-dependent event that is of relevance is the acquisition of a functional FDC network within the germinal centers of peripheral lymphoid tissue.

The second phase of neuroinvasion appears to encompass transfer of prions from lymphoid tissue to nerve endings of the PNS. Because lymphoid organs are predominantly innervated by nerve fibers of the SNS, this part of the SNS

is a prime candidate. How neuroinvasion is accomplished, and how the agent is transported within the PNS however are still unclear. It is worthwhile noting that the innervation of lymphoid tissue is, at least in part, controlled by lymphocytes themselves, because both T- and B-cells secrete nerve growth factor and, vice versa nerve terminals secrete a variety of factors that stimulate the immune system ***(74)***. These factors may play a critical role in the neuroinvasion process and represent a critical site for modulation of disease progression. For example, drugs that act on lymphocytes or on the sympathetic innervation of lymphoid tissue, or those that prevent cytokine release or block neurotransmission, may have a strong influence in the immune modulation, and may represent useful tools for studying the cellular and molecular basis of prion neuroinvasion.

Acknowledgments

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9

Cellular and Transgenic Models of Familial Prion Diseases

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1. Introduction

Prion diseases are fatal neurodegenerative disorders of humans and animals, which result from the conformational conversion of a normal, cell surface glycoprotein (PrP^{C}) into a pathogenic isoform (PrP^{Sc}) that is the main component of infectious prions ***(1,2)***. Familial prion diseases, which include 10% of the cases of Creutzfeldt-Jakob disease and all cases of Gerstmann-Sträussler syndrome and fatal familial insomnia, are linked in an autosomal dominant fashion to point and insertional mutations in the PrP gene on chromosome 20 ***(3,4)***. These mutations are presumed to favor spontaneous conversion of PrP to the PrP^{Sc} state. One way to experimentally model familial prion diseases is to express PrP molecules carrying disease-associated mutations in either cultured mammalian cells or transgenic mice. The authors review their own work using these two kinds of model systems, which have provided complementary information about the $PrP^{C} \rightarrow PrP^{Sc}$ conversion process, and about the pathogenic effects of mutant PrP.

2. Cultured Cells Convert Mutant PrPs to a PrP^{Sc}-Like State

We have created stably transfected lines of Chinese hamster ovary (CHO) cells that express mouse PrP (moPrP) molecules carrying mutations homologous to seven different pathogenic mutations of humans. As a negative control, cells have also been analyzed that express moPrP with a substitution of valine for methionine at codon 128, homologous to a nonpathogenic polymorphism at codon 129 in human PrP.

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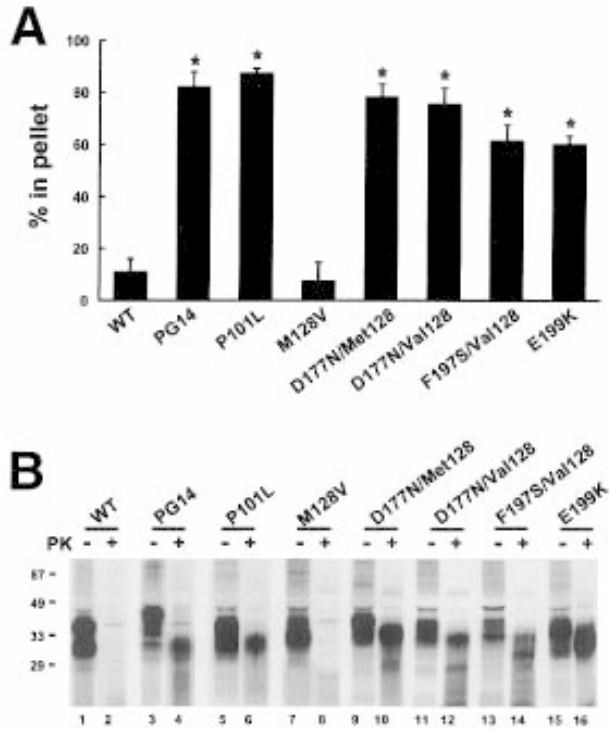
The authors find that moPrPs carrying pathogenic mutations acquire all of the biochemical hallmarks of PrP^{Sc} ***(5–7)***. PrP^{Sc} can be distinguished operationally from PrP^{C} by several biochemical properties. One commonly used property is insolubility in nondenaturing detergents, which can be assayed by subjecting detergent lysates to ultracentrifugation. Although most of the wild-type PrP remained in the supernatant under these conditions, the majority of each of the PrPs carrying a pathogenic mutation was found in the pellet (**Fig. 1A**). As expected, M128V moPrP behaved like the wild-type protein. A second characteristic of PrP^{Sc} is resistance to proteolysis, which is manifested by production of protease-resistant core fragment (PrP 27–30) upon treatment with proteinase K. Treatment of the mutant PrPs with 3.3 μg/mL proteinase K for 10 min resulted in production of a PrP27–30 fragment; under the same conditions wild-type and M128V moPrPs were completely digested (**Fig. 1B**). Immunoblotting with sequence-specific antibodies demonstrated that the PrP27–30, fragment is cleaved just after the octapeptide repeats, which is the same region where authentic PrP^{Sc} is cleaved.

Analysis of transfected CHO cells expressing mutant PrPs has provided several important insights into the cell biology of PrP^{Sc}, as described below.

3. Membrance Topology of Mutant PrP^{Sc}

PrP^{C} is attached to cell membranes by a C-terminal glycosylphosphatidylinositol (GPI) anchor, and can be released by cleavage of the anchor using bacterial phosphatidylinositol-specific phospholipase C (PIPLC). The nature of the membrane attachment of PrP^{Sc}, however, has been a matter of uncertainty. Chemical analysis of the purified protein demonstrates that PrP^{Sc} possesses a C-terminal GPI anchor ***(8)***. Unlike PrP^{C}, however, PrP^{Sc} is not releasable by PIPLC from brain membranes or from the surface of scrapie-infected N2A cells ***(5,9–11)***.

Fig. 1. (*Opposite page*) PrPs carrying disease-related mutations are detergent-insoluble and protease-resistant when expressed in cultured CHO cells. (**A**) CHO cells expressing wild-type and mutant mouse PrPs were labeled with [^{35}S]methionine for 20 minutes and then chased for 3 hours. Detergent lysates of the cells were centrifuged first at 16,000 *g* for 5 min, and then at 265,000*g* for 40 min. PrP in the supernatants and pellets from the second centrifugation was immunoprecipitated and analyzed by SDS-PAGE. PrP-specific bands were quantitated using a Phosphor-Imager, and the percentage of PrP in the pellet was calculated. Each bar represents the mean ±SD of values from three experiments. Values that are significantly different from wild type PrP by t-test ($p < 0.001$) are indicated by an asterisk. PrPs carrying disease-related mutations sediment (are detergent-insoluble), while wild type and M128V PrPs remain



largely in the supernatant. Human homologues of the mutant PrPs analyzed here are associated with the following phenotypes: PG14 (9-octapeptide insertion), CJD-variant; P101L, GSS; M128V, normal; D177N/Met128, FFI; D177N/Val128, CJD; F197S/Val128, GSS; E199K, CJD. (**B**) CHO cells expressing each PrP were labeled for 3 h with [^{35}S]methionine, and chased for 4 hours. Proteins in cell lysates were either digested at 37°C for 10 minutes with 3.3 μg/ml of proteinase K (+ lanes), or were untreated (- lanes), prior to recovery of PrP by immunoprecipitation. Five times as many cell-equivalents were loaded in the + lanes as in the - lanes. Molecular weight markers are in kilodaltons. PrPs carrying pathogenic mutations yield a protease-resistant fragment of 27-30 kDa, while wild type and M128V PrPs are completely degraded. Modified with permission from **ref. *31***.

Our analysis of mutant PrPs in cultured CHO cells has shed new light on the issue of the membrane attachment of PrP^{Sc}. Mutant PrPs, like PrP^{Sc} from infected brain, are not released from membranes by treatment with PIPLC ***(12)***. This property does not result from absence of a GPI anchor structure, because the mutant PrPs metabolically incorporate the anchor precursors [^{3}H]ethanolamine, [^{3}H]palmitate, and [^{3}H]stearate. Although the authors originally postulated that mutant PrPs possessed a secondary mechanism of mem-

brane attachment, in addition to their GPI anchors *(12)*, more recent evidence suggests that the mutant molecules are resistant to PIPLC release, because their GPI anchors become physically inaccessible to the phospholipase as part of their conversion to the PrP^{Sc} state *(13)*. This conclusion is based on failure of PIPLC to quantitatively remove [^{3}H]palmitate label from the proteins, or to render them hydrophilic by Triton X-114 phase partitioning. Resistance to cleavage is observed when PIPLC is applied to intact cells, as well as when treatment is carried out after lysis in nondenaturing buffers. However, denaturation in sodium dodecyl sulfate renders the GPI anchor of the mutant PrPs susceptible to cleavage, suggesting that PIPLC-resistance depends on the native structure of the protein. We now view PIPLC resistance as being an operational property analogous to protease-resistance, and postulate that it reflects an alteration in the structure of PrP attendant on conversion to the PrP^{Sc} state.

Although most PrP^{C} molecules are attached to the cell membrane exclusively by a GPI anchor, it has been reported that there exists a subpopulation that displays a transmembrane orientation. Two transmembrane species of PrP have been identified, each with the same membrane-spanning segment (residues 112–135), but with opposite orientations of the polypeptide chain *(14,15)*. It has recently been suggested *(16)* that one of these species (^{Ctm}PrP, which has its C-terminus in the endoplasmic reticulun [ER] lumen) is the primary effector of neurodegeneration in both inherited and infectious cases of prion diseases, since mice carrying certain mutations in the transmembrane region, as well as those infected with scrapie, showed increased amounts of C^{tm}PrP *(16)*. In contrast, our own results suggest that ^{Ctm}PrP is unlikely to be part of a general pathogenic pathway, because disease-associated mutations outside of the transmembrane domain do not increase the amount of this form *(17)*.

4. Kinetics and Subcellular Localization of Mutant PrPSc Synthesis

We have used transfected CHO cells to identify intermediate biochemical steps in the conversion of mutant PrPs to the PrP^{Sc} state *(18)*. The strategy was to measure the kinetics with which three PrP^{Sc}-related properties (PIPLC resistance, detergent insolubility, and protease resistance) develop in pulse–chase labeling experiments. This has allowed definition of three steps in the conversion process (**Fig. 2**). The earliest biochemical change that could be detected in mutant PrP, one that was observable within minutes of pulse-labeling cells, was the acquisition of PIPLC resistance, a property that was revealed by partitioning of the phospholipase-treated protein into the detergent phase of Triton X-114 lysates, or by its binding to phenyl-Sepharose. The second step is acquisition of detergent insolubility, which is not maximal until 1 h of chase *(6)*,

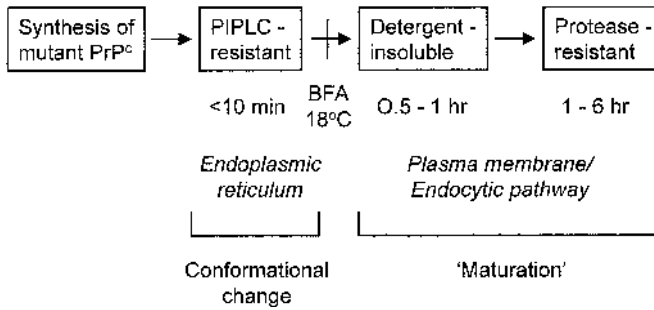


Fig. 2. A scheme for transformation of mutant PrPs to a PrP^{Sc} state. Mutant PrPs are initially synthesized in the PrP^{C} state, and acquire PrP^{Sc} properties in a stepwise fashion as they traverse different cellular compartments. PIPLC-resistance, which develops in the ER, reflects folding of the polypeptide chain into the PrP^{Sc} conformation. Detergent-insolubility and protease-resistance, which develop upon arrival at the plasma membrane or along an endocytic pathway, result from intermolecular aggregation ("maturation"). The times given underneath the boxes indicate when after pulse-labeling the corresponding property is detected. Addition of brefeldin A (BFA) to cells or incubation at 18°C, treatments which block movement of proteins beyond the Golgi apparatus, inhibit acquisition of detergent-insolubility and protease-resistance but not PIPLC-resistance. Modified with permission from **ref. *18***.

suggesting that it occurs after the acquisition of PIPLC-resistance. Detergent insolubility presumably reflects aggregation of PrP molecules, and by sucrose gradient fractionation aggregates were detected ranging in size from 4S (monomeric) to over 20S (>30 PrP molecules). The third step is acquisition of protease-resistance, which is not maximal until several hours after labeling ***(6)***. We have hypothesized that the fundamental conformational change that underlies conversion of mutant PrP^{C} into PrP^{Sc} is reflected in the acquisition of PIPLC resistance, with detergent insolubility and protease resistance being secondary properties that develop sometime after the initial molecular conversion.

Our kinetic studies suggest that individual steps in the formation of PrP^{Sc} may take place in at least two different cellular locations (**Fig. 2**). Because mutant PrPs become PIPLC-resistant within minutes of synthesis in pulse-labeling experiments, this early step must take place in the ER. Consistent with this conclusion, acquisition of PIPLC resistance is not affected by treatment of cells with brefeldin A, or by incubation at 18ºC, which are manipulations that block exit of proteins beyond the Golgi ***(18)***. In contrast, detergent insolubility and protease resistance, which do not develop until later times of chase, and are reduced by brefeldin A and 18ºC incubation, are likely to be acquired after arrival of the protein at the cell surface, either on the plasma membrane itself or in endocytic compartments. The idea that the generation of PrP^{Sc} from mutant PrPs may begin in the ER is theoretically appealing, because of the well known role of this

organelle in protein folding. It is also reasonable to suggest that ER chaperones are good candidates for the hypothetical cellular co-factors that are widely thought to play an important regulatory role in prion synthesis ***(19)***.

5. Generation of Tg Mice Expressing a Mutant PrP

To extend the cell culture work, and to investigate the properties of mutant PrP in an in vivo setting, we constructed mice bearing a moPrP transgene that contains a nine-octapeptide insertional mutation ***(20,21)***. The human homolog of this mutation, which is the largest insertion thus far described in the PrP gene, has been found in two patients (one British and one German) who were afflicted with an illness characterized by progressive dementia and ataxia, and, in the one autopsied case, by the presence of PrP-containing amyloid plaques in the cerebellum and basal ganglia ***(22–24)***. The transgenic mice model key clinical and neuropathological features of human familial prion diseases, and unlike other mice harboring PrP transgenes ***(14,25–28)***, they spontaneously accumulate PrP^{Sc} in their brains. Analysis of these mice has provided important insights into the natural history and pathogenesis of familial prion diseases.

5.1. Neurological Symptoms of Tg(PG14) Mice

Tg(PG14) mice from the A2 and A3 lines (both of which express mutant PrP at levels similar to that of endogenous PrP) develop a progressive and ultimately fatal neurological disorder characterized by ataxia, kyphosis, foot-clasp reflex, waddling gait, difficulty righting, and weight loss (**Fig. 3**; ***20,21***). Similar symptoms were also observed in a founder (A1) that did not breed, and that expressed PG14 PrP at 4× the level of endogenous PrP. In contrast, Tg(WT) mice which express wild-type PrP at even higher levels (3–4× endogenous PrP), remain healthy. We observed that breeding the transgene array to homozygosity dramatically accelerated the onset of disease (from 235 ± 10 to 68 ± 9 d of age), and shortened its duration (from 154 ± 14 to 49 ± 11 d). This effect is probably attributable to the twofold higher expression of PG14 PrP in homozygous, compared to heterozygous, mice. This explanation is consistent with our finding that the Tg(PG14) B and C lines, which express low levels of the mutant protein (15% of the endogenous PrP level), do not develop a neurological disorder within the lifespan of the animals. Taken together, these results indicate that overexpression of wild-type PrP does not produce neurological dysfunction, and that development of the disease in Tg(PG14) mice is related to the expression level of the mutant protein.

5.2. Neuropathological Abnormalities in Tg(PG14) Mice

Several pathological changes were observed in the A2 and A3 lines of T(PG14) mice, and in the A1 founder ***(20,21)***. The most obvious was a massive degenera-

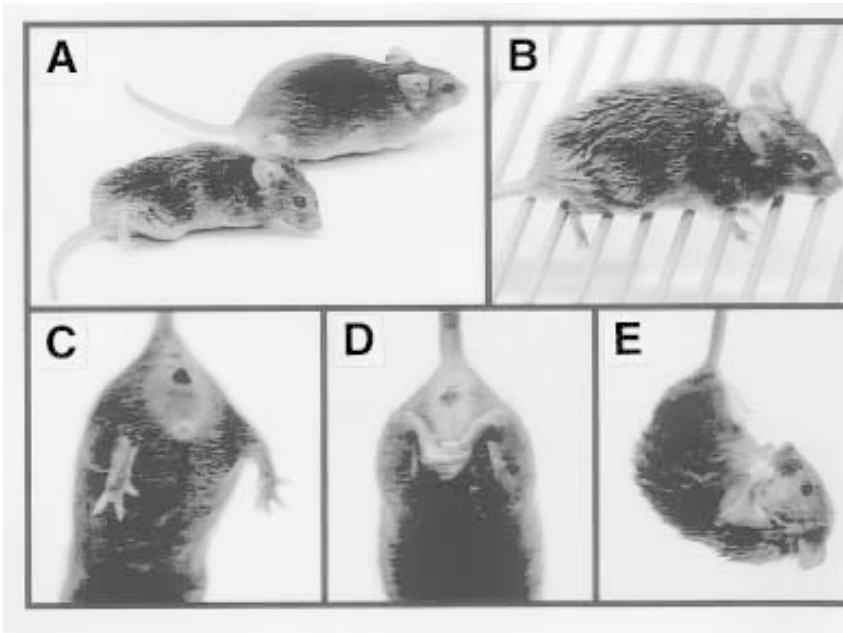


Figure 3. (**A**) Neurological symptoms in Tg(PG14) mice. (**A**) Tg(PG14-A3)/Prn-p0/0 mouse at 84 days of age (left), and Tg(WT-E1)/Prn-p0/0 mouse at 89 days of age. Note the ataxic posture of the PG14 mouse with hind limbs extended, the hunchback orientation of the body, and the ruffled appearance of the coat. (**B**) The Tg(PG14-A1) founder at 319 days of age is completely incapable of ambulating on a metal grill. Normal mice walk easily on the grill and rarely let their feet slip through the bars. (**C**) When suspended by its tail, a Tg(WT) mouse, like a nontransgenic mouse, splays its hind limbs apart. (**D**) In contrast, the Tg(PG14-A3) founder at 324 days of age tightly clasps its hind limbs together. (**E**) At 84 days of age, a Tg(PG14-A3)/Prn-p0/0 mouse suspended by its tail assumes a flexed posture, attempting to clasp all four limbs together. Reproduced with permission from *ref.* ***20.***

tion of cerebellar granule cells, which begins by 30 d of age in Tg($PG14^{+/+}$) mice, and eventually results in severe atrophy of the cerebellum (**Fig. 4A–D**). Several features indicate that, in Tg(PG14) mice, degeneration of granule cells occurs by an apoptotic mechanism, these including the presence of numerous pyknotic and fragmented granule cell nuclei, positive staining of degenerating neurons, both by *in situ* end-labeling of DNA and by an antibody to activated caspase-3, and the presence in cerebellar DNA preparations of a 200-bp ladder indicative of internucleosomal cleavage. Tg(PG14) mice thus provide a particularly clear-cut demonstration of the role of apoptosis in a prion disease.

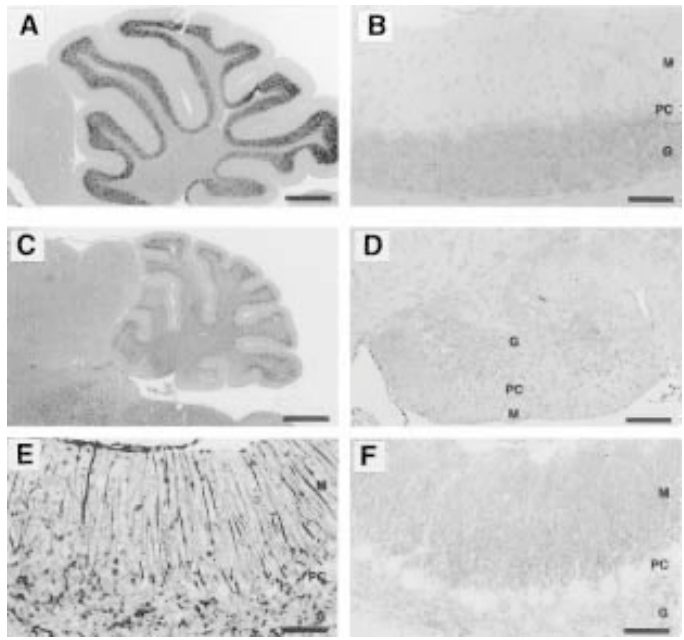


Figure 4. Neuropathological findings in the cerebella of Tg(PG14) mice. (**A**) Cerebellum of a healthy Tg(WT-E1)/Prn-p0/0 mouse at 184 days of age appears normal after staining with hematoxylin and eosin. (**B**) Cerebellar cortex of the same mouse shown in panel A, stained with antibody 3F4, shows no deposits of PrP, and normal appearance of the molecular (M), Purkinje cell (PC) and granule cell (**G**) layers. (**C**) Cerebellum of a terminally ill Tg(PG14-A3$^{+/+}$)/Prn-p0/0 mouse at 183 days of age after staining with hematoxylin and eosin. There is marked atrophy of cerebellum, with reduction in the thickness of the granule cell and molecular layers. (**D**) Cerebellar cortex of the same mouse shown in panel C, stained with antibody 3F4. There is a dramatic reduction in the density of granule cells, and relatively weak staining for PrP. (**E**) Cerebellar cortex of the symptomatic Tg(PG14-A1) founder at 319 days of age, stained for GFAP. There is marked hypertrophy of Bergmann glial fibers in the molecular layer, and increased numbers of astrocytes in the granule cell layer. (**F**) Cerebellar cortex of a moderately symptomatic Tg(PG14-A3$^{+/+}$)/Prn-p0/0 mouse at 71 days of age, stained with antibody 3F4. There is heavy PrP deposition in a synaptic-like pattern in the molecular layer, and less prominent staining in the granule cell layer. Purkinje cells are unstained, since the transgenic vector does not drive expression in this cell type. PrP staining is more intense in this mouse compared to the older animal shown in panel D, since the granule cell layer is more intact in this animal, and granule cells are an important source of PrP. Scale bars are 556 μm (**A** and **C**), 97 μm (**B** and **D**), 44 μm (**E**), and 62 μm (**F**). Reproduced with permission from *ref.* ***20.***

A second abnormal feature in Tg(PG14) mice was the presence of punctate, "synaptic-like" deposits of PrP, which were most prominent in the cerebellum (**Fig. 4F**), hippocampal formation, and olfactory bulb, and were present to a lesser extent in the neocortex and inferior colliculus. A third finding was astrocytic gliosis, which was observed in the cerebellar cortex (**Fig. 4E**), the hippocampus, and the neocortex. PrP deposition and gliosis began at 30–40 d of age in Tg(PG14$^{+/+}$) mice, and increased as the illness progressed. Neither thioflavin-positive plaques, nor obvious spongiosis were observed at any time. No pathological changes were seen in Tg(WT) or non-transgenic mice.

5.3. PrP^{Sc}-Like Form of PrP in Tg(PG14) Mice

PG14 PrP in the brains of Tg(PG14) mice displays the same biochemical hallmarks of PrP^{Sc} that we observed for mutant PrPs in CHO cells, including detergent insolubility (**Fig. 5A**), protease resistance (**Fig. 5B–D**), and resistance of the GPI anchor to cleavage by PIPLC ***(20,21)***. Detergent-insoluble and protease-resistant PG14 PrP is already synthesized in the brains of transgenic mice during the first week of life, well before the animals develop clinical symptoms or neuropathological changes ***(20,21)***. Moreover, the amount of detergent-insoluble and protease-resistant PrP increased dramatically with age, with levels in the oldest, terminally ill animals that were up to 80-fold higher than in newborn mice. PrP^{Sc}-like protein accumulated more rapidly and to higher levels in Tg(PG14$^{+/+}$) mice than in Tg(PG14$^{+/-}$) mice, which correlates with the accelerated disease progression in the homozygous animals. These results indicate that mutant PrP is converted continuously to the PrP^{Sc} state throughout life, but that clinical symptoms and neuropathological lesions do not ensue until the amount of PrP^{Sc} reaches a critical threshold level. If the same is true in human patients, this would explain why familial prion diseases do not manifest themselves clinically until adulthood, even though mutant PrP is probably synthesized beginning before birth ***(29)***.

To assess the neuroanatomical distribution of the PrP^{Sc}-like form of PG14 PrP, we carried out histoblots of cryostat sections of brain ***(30)***. Protease-resistant PrP was found throughout the brains of both preclinical and terminally ill Tg(PG14) mice, with particular concentrations in the medial caudate-putamen, septum, corpus callosum, anterior commissure, ventral thalamus, globus pallidus, and hippocampus ***(21)***. The widespread anatomical distribution of the PrP^{Sc} revealed by these methods contrasts with the more restricted distribution of punctate PrP deposits seen by immunohistochemistry (see above), raising the possibility that PrP^{Sc} may be more aggregated in certain regions, such as the cerebellum. Surprisingly, we have also found a detergent-insoluble and protease-resistant form of PG14 PrP in a number of the peripheral tissues in which PrP is normally expressed (although at lower levels than in brain), including skeletal muscle, heart, kidney, and testis ***(21)***.

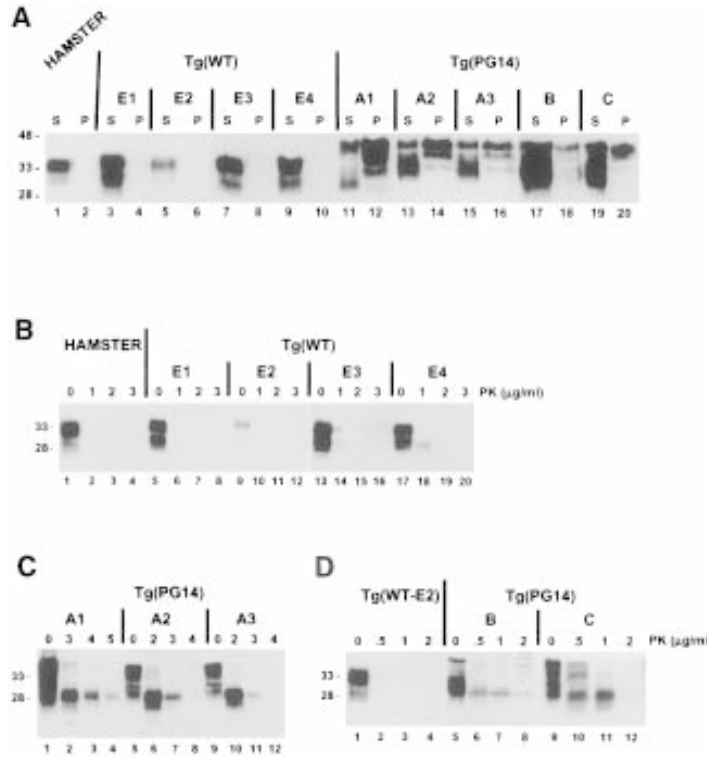


Figure 5. PG14 PrP in the brains of transgenic mice is detergent-insoluble and protease-resistant. (**A**) Detergent lysates of brain (containing 100 μg of protein) from transgenic mice and from Syrian hamster were centrifuged at 260,000 X g for 40 min. Proteins in supernatants (S lanes) and pellets (P lanes) were then separated by SDS-PAGE and immunoblotted using antibody 3F4. One-fourth of each sample (lanes 1-16), or the whole sample (lanes 17-20) was run on the gel. (**B-D**) Detergent lysates of brain from transgenic mice and Syrian hamster were incubated with the indicated amounts of PK for 30 min at 37°C. Digestion was terminated by addition of PMSF, and methanol-precipitated proteins were separated by SDS-PAGE and immunoblotted using antibody 3F4. 200 μg (**B** and **C**) or 800 μg (**D**) of initial protein was subjected to digestion. The lanes containing undigested samples (0 μg/ml PK) represent 50 μg (**B** and **C**) or 200 μg (**D**) of protein. Mice were of the following ages and clinical status at the time of sacrifice: E1-E4, 203 days old (all healthy); A1, 319 days old (severely symptomatic); A2, 71 days old (healthy); A3, 205 days old (mildly symptomatic); B, 169 days old (healthy); C, 226 days old (healthy). Modified with permission from *ref.* ***20.***

6. Conclusions

We have analyzed two complementary experimental systems for expression of mutant PrP molecules carrying disease-associated mutations, one based on transfected cells in culture, and one based on transgenic mice. Both systems convert mutant PrP molecules to a PrP^{Sc}-like form, the biochemical properties of which are remarkably similar in the two systems, which suggests that structural features of the mutant PrPs themselves, perhaps in conjunction with ubiquitous cellular co-factors, are the primary determinants of the conversion reaction. It is noteworthy that the mutant PrPs from transgenic mice and CHO cells differ from authentic PrP^{Sc} in their considerably lower level of protease resistance ***(5,20)***, suggesting that they represent a biochemical intermediate along the pathway from PrP^{C} to PrP^{Sc}. This intermediate has presumably acquired some of the biochemical features of PrP^{Sc}, but is structurally distinct, perhaps because it has only partial β-sheet character, or is less aggregated or polymerized. An important test will be to determine whether mutant PrPs from cells and Tg mice are infectious in animal bioassays, which is an experiment currently underway. Regardless of the results, however, the fact that the mutant proteins possess other PrP^{Sc}-like properties in addition to protease resistance, and the fact that they produce a neurological illness in vivo, make it likely that at least some aspects of PrP^{Sc} formation are being modeled in both CHO cells and Tg(PG14) mice. Thus, the insights gained from these systems significantly advance understanding of prion diseases on both a cell biological and pathophysiological level.

Acknowledgments

This work was supported by grants to D.A.H. from the National Institute of Health, the American Health Assistance Foundation, and the Alzheimer's Association, and to B.G. from the NIH. R.C. was the recipient of fellowships from the Comitato Telethon Fondazione Onlus, and the McDonnell Center for Cellular and Molecular Neurobiology at Washington University.

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10

Central Nervous System Inflammation and Prion Disease Pathogenesis

Samar Betmouni and V. Hugh Perry

1. Introduction

The study of inflammation in the prion diseases is relatively new. Indeed, for a number of years the accepted dogma was that the prion diseases lacked an inflammatory response in the brain ***(1–3)***. This persists in spite of a number of studies showing that the pathological hallmarks of the prion diseases (PrP^{Sc} deposition, astrocytosis, vacuolation, and neuronal loss) are associated with the presence of activated microglia ***(4–7)***. At the heart of this discrepancy is a simple matter of what is meant by inflammation. The innate inflammatory response is the tissue's response to injury or infection, and, as so succinctly put by Metchnikoff in the late nineteenth century, "The essential and primary element in typical inflammation consists in a reaction of the phagocytes against a harmful agent" ***(8)***. Given that the microglia are the brain's resident macrophages (i.e., phagocytic cells), we believe that the presence of activated microglia in prion-affected brains represents an inflammatory response ***(9–11)***.

Another problem that arises is one of histological description. The histological characteristics of inflammation include perivascular cuffing of neutrophils, in the case of an acute innate inflammatory response, or lymphocytes, plasma cells, and macrophages, in the case of chronic immune inflammation. An acute response in the periphery (e.g., skin) is characterized by the presence of neutrophils (by 6–12 h) and later monocytes/macrophages (12–24 h) at the site of injury, but the brain's response is altogether different ***(12–14)***. Following either a pro-inflammatory (e.g., lipopolysaccharide, cytokines) or a neurotoxic (e.g., kainic acid) lesion to the brain parenchyma, in addition to the activation of the resident microglia, there are few neutrophils and a delay in

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monocyte response by 48–72 h. Therefore, the acute inflammatory response in the brain is different than that seen in the periphery, because there is no leucocyte cuffing around blood vessels in the injured brain parenchyma. It is, however, identical to a peripheral response, in as much as it functions to activate and deliver phagocytic cells to the site of injury.

Unlike the prion diseases, the association of central nervous system (CNS) inflammation in Alzheimer's disease as characterized by the presence of activated microglia, is now well accepted ***(15,16)***. In light of the similarities between Alzheimer's disease and the prion diseases ***(17)***, it would be surprising to find that CNS inflammation was not also a component of the prion diseases.

2. Evidence for an Inflammatory Response in the Prion Diseases

Conspicuous activation of microglia has been shown at the terminal stages of a number of experimental models of prion disease ***(4,18)***. The distribution of activated microglia correlated directly with the distribution of PrP^{Sc} amyloid plaque deposition and vacuolation. Activated microglia have also been seen in association with PrP^{Sc} amyloid plaques in the human prion diseases including Kuru, Creutzfeldt-Jakob disease (CJD), and Gerstmann-Sträussler-Scheincke disease ***(3,5,6,19–21)***; however it is not clear what, if any, role the inflammatory response observed in the brain at advanced stages of both human and experimental prion disease, plays in the pathogenesis of this group of diseases. In order to address this question, we have carried out studies to characterize the inflammatory cells at earlier time points during the course of the disease, and to define the inflammatory pathways that are activated in these cells.

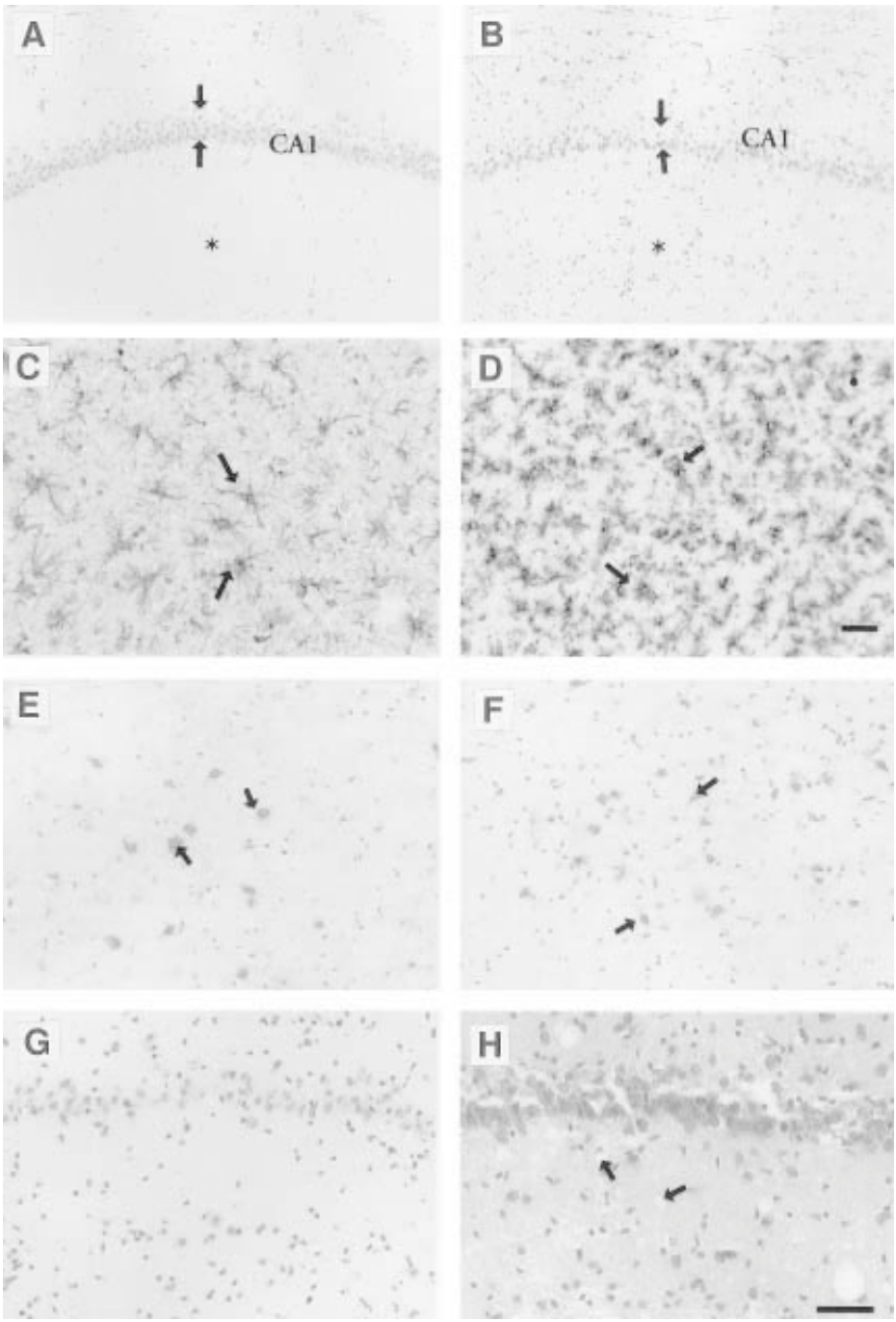
3. Experimental Model

We have studied in detail one strain of prion agent, the ME7 strain of scrapie, which is in many respects the archetype of murine prion agents. It has been studied for many years, and produces a well-defined but widespread pathology that includes neuronal loss, astrocyte activation, amyloid deposits of PrP^{Sc}, and microglial activation ***(4,22–25)***. We routinely use stereotaxic microinjection (1 μL) of either prion-affected (ME7 strain of scrapie) or normal brain homogenate (NBH) into the dorsal hippocampus of C57BL/6J mice. In addition to minimizing the trauma that is probably associated with the injection of larger volumes of inoculum, focal microinjection has allowed exploitation of one of the well-documented properties of the prion agent to spread within the CNS along neuroanatomical connections ***(26,27)***. In this way we have been able to follow the progress of the disease as it spreads from its origin in the dorsal hippocampus, first to the contralateral hippocampus and also rostrally into the septum and diagonal band of Broca, along well-described neuronal pathways ***(28)***.

It has been possible to reproduce the previously reported clinical and pathological features of mouse scrapie ***(29)***, using focal microinjection. The mice display the typical clinical signs of disease, including hunched posture, piloerection, and reduction in mobility, at 23 wk after injection with ME7, and it is a further 2–3 wk before the mice become more profoundly ill ***(7)***. In addition, we have been able to confirm the presence of astrocytosis, neuronal loss, and PrP^{Sc} deposition and vacuolation at end-stage disease (**Fig. 1**). The vacuolation, visible as holes in the tissue with a diameter of 5–15 μm, is, however, largely an artifact of tissue processing ***(30)***. Although this was first reported in the late 1960s ***(31)***, it has been overlooked more recently, which is surprising, because the pattern of vacuolation, or "lesion profile," has played an important role in defining the different prion strains, including variant (v)CJD ***(32)***.

The vacuolation is only a feature of fixed, paraffin-embedded brains, and is not seen on fresh-frozen cryostat sections. This artifact ***(30)*** may have implications for the understanding of prion disease pathogenesis. The vacuoles do not appear to contain lipid or carbohydrate storage material, and they do not appear to be lysosomal in origin. Because vacuolation is a feature of paraffin-embedded tissue, it is likely that the vacuoles arise as a consequence of tissue shrinkage, which occurs during paraffin processing, and this may relate to an alteration in the brain's extracellular matrix. We thus looked for evidence of increased matrix metalloproteinase activity that might be involved in extracellular degradation. The 25-fold increase in stromelysin-1 mRNA expression, which is present at end-stage disease in this model, suggests that there is some modeling or degradation of the brain's extracellular matrix and that this in turn could contribute to neuronal degeneration. There is evidence from the work of Chen and Strickland ***(33)*** that the interaction between components of the brain's extracellular matrix and neurons is neuroprotective, and that disruption of this interaction by proteases directly contributes to the neuronal death in acute neurotoxic models.

We have also shown that there is an upregulation of a nonnuclear isoform of histone H1 in the brains of prion-affected mice with clinical signs of disease ***(34)***. This is seen both on Western blots, and on immunocytochemistry, in which upregulation of H1 is seen in neurons and within the neuropil and astrocytes in areas of the brain affected by prion pathology. A similar picture is seen in Alzheimer's disease brains. Histone H1, a lipopolysaccharide-binding protein, is a cell surface protein that is constitutively expressed on neurons. The role of this neuronal isoform is not established, but it has been suggested that it may act as an acute-phase protein ***(35)***, and that it may have antibacterial properties ***(36)***. Its role in the pathogenesis of prion disease requires further study. The absence of histone H1 upregulation in models of acute CNS inflammation ***(34)*** suggests that either the chronic neurodegenerative process and/or PrP^{Sc} accumulation is the trigger for histone H1 upregulation in prion disease.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4. The Inflammatory Response

We have demonstrated the presence of activated microglia at end-stage disease, and, in addition, were the first to show the presence of cytotoxic ($CD8^+$) T-lymphocytes in prion-affected brains *(7)*. The distribution of $CD8^+$ T-cells coincides with the distribution of activated microglia (**Fig. 2**). Since, at the end-stage of murine prion disease, there is massive neuronal degeneration, the presence of activated microglia is hardly surprising. The key issue is when does microglial activation first occur in the disease process, and how can microglial activation in prion pathogenesis be separated from the inflammation likely to be induced by the delivery of the brain homogenate to the brain parenchyma. We have found that there is little to distinguish between the acute inflammatory response in the CNS after the injection of ME7 or NBH up to 4 wk after intracerebral challenge *(37)*. In the region of the injection site, the number and distribution of activated microglia and $CD8^+$ T-cells was similar, comprising a peak of microglial and T-cell responses at 2–5 and 2–7 d respectively. In both cases, the blood–brain barrier was restored by 1 wk postinjection, and the acute inflammatory response had completely resolved by 4 wk. It is most likely, therefore, that the acute CNS response is secondary to the surgical procedure, and, more generally, to the injection of brain homogenate. There are additional factors that contribute to the neurodegeneration that is only seen later in ME7-injected mice.

An interesting point that arose from the study of the acute events following the injection of the brain homogenates was that it was not possible to attribute any acute neurotoxic action to ME7. There are studies that implicate fibrillogenic fragments of PrP^{Sc} in the neurotoxicity observed in in vitro models *(38–40)*, but the evidence that PrP^{Sc} is neurotoxic in vivo remains circum-

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Fig. 1. Typical neuropathology seen in sections of brain taken from prion-affected mice with established clinical signs of disease (incubation period). Note the reduced thickness of the pyramidal cell layer (arrow heads) and the increased cellularity of the surrounding parenchyma (*), which is typical of sections of brain taken from prion-affected mice **(B)**, compared with age- and time-matched controls injected with normal brain homogenate **(A)**. The neuronal loss is associated with a marked astrocytosis, as seen using glial fibrillary acidic protein immunocytochemistry **(C)** and conspicuous microglial activation **(D)**, as seen with an antibody to CD68. Consecutive sections show that the areas of microglial activation are also associated with the presence of $CD3^+$ T-cells, using the pan T-cell marker KT3 **(E)**, almost all of which belong to the $CD8^+$ cytotoxic T-cell subset **(F)**. Vacuolation is not observed in fresh-frozen sections of brain **(G)**, but is visible on fixed, paraffin-embedded tissue **(H)**. Both G and H are from ME7-injected mice with clinical signs of disease. Scale bars = 50 μm

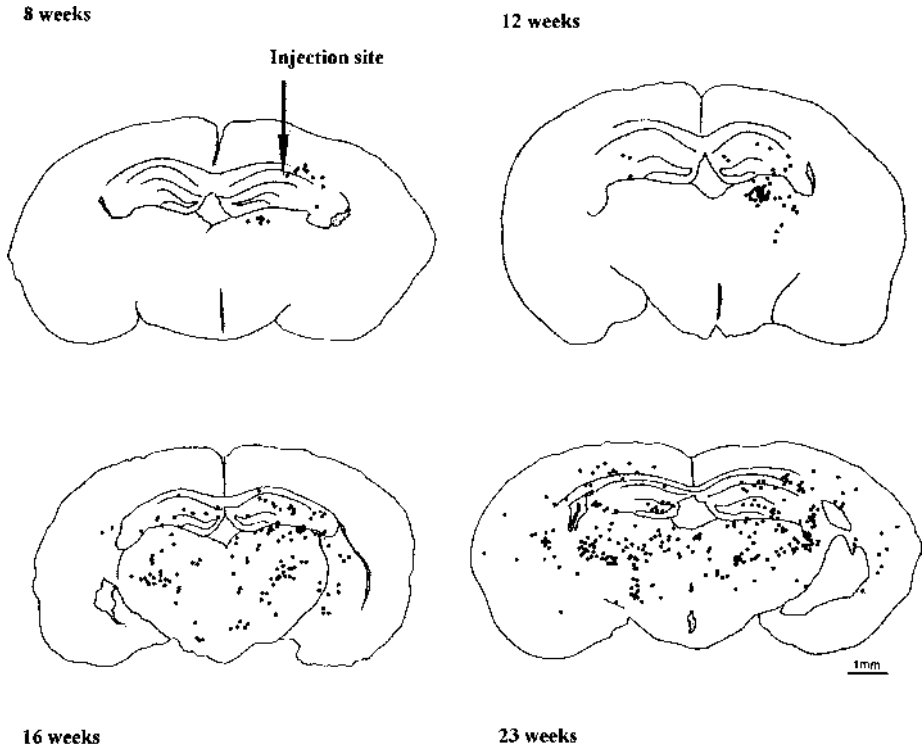

Fig. 2. The natural history of T-cell distribution in murine prion disease. Diagram illustrating the progress of $CD8^+$ T-cell distribution at the different survival times indicated, following the injection of ME7 brain homogenate. The representative coronal sections at the level of the injection site were drawn using a microscope drawing tube. Each drawing shows the distribution in a single 10-μm section; each dot represents the location of one $CD8^+$ T-cell. This distribution of $CD8^+$ T-cells is similar to that of activated microglia at corresponding time-points during the course of the disease.

stantial. Indeed, although PrP^{Sc} co-localizes with areas of vacuolation and astrocytosis ***(41,42)***, there are reports of cases in which the neuropathology of prion disease occurs in the absence of PrP^{Sc} accumulation ***(43)***.

The earliest evidence of pathology that can be attributed to the injection of ME7, as distinct from the injection of NBH, is seen at 8 wk and comprises the co-distribution of activated microglia and $CD8^+$ T-cells in close association with the injection site. There is no ongoing neuronal cell loss in the dorsal hippocampus at this time-point, so it is likely that this well-circumscribed inflammatory response is secondary either to the local accumulation of PrP^{Sc} or to subtle neuronal dysfunction caused by the accumulation of PrP^{Sc}. Because microglia respond to almost any disturbance of brain homeostasis ***(44)***, the microglial acti-

vation informs us that pathology is initiated between 4 and 8 wk. The pathology triggers an inflammatory response that is highly atypical, and, once this has happened, a stereotyped process ensues, resulting in the predictable spread of pathology to anatomically connected regions of the brain. This process culminates in the development of overt neuronal loss by 20 wk. After this time, the health status of the ME7-injected mice begins to deteriorate, as expressed first by a reduction in muscle strength ***(45)*** and a progressive decline in body weight, and, up to 4 wk later, by the development of the typical clinical signs of disease.

In spite of the marked inflammatory response from at least halfway through the course of the disease, indicating an ongoing pathological process, the mice appear to be well on routine monitoring of health status. It is not until the mice are examined more closely, using a panel of behavioral tests, that it is possible to detect subtle changes in behavior. The targeting of the hippocampus by direct focal injection, and the use of a scrapie strain (ME7) known to cause hippocampal pathology ***(46)***, opened the way to the use of behavioral tests that are sensitive to hippocampal lesioning. Hippocampal lesions are known to produce locomotor hyperactivity and impairment in passive avoidance behaviors ***(47)***; the use of open-field tests and multitrial passive avoidance, respectively, has enabled the detection of subtle changes in behavior during the early stages of prion disease. We have shown that prion-affected mice have behavioral changes from as early as 12 wk when locomotor hyperactivity is present, and, at 14 wk, when there is an impairment of cognition on the passive-avoidance task ***(45)***.

The few studies that have investigated the temporal development of behavioral changes in murine prion disease have produced conflicting results ***(48–53)***. These differences may relate to different mouse strain–scrapie-agent strain combinations, different routes of delivery, and different injection volumes used in these experiments. Our findings are similar to those of McFarland et al. ***(51)***, who, although using a different mouse strain–agent strain combination, showed that there was generalized hyperactivity in scrapie-affected mice, from approximately 60% of the way through the incubation period. They did not, however, comment on the activity of the mice at later stages of the disease. Another study, also using a different mouse strain–agent strain combination, reported that there was a reduction in the locomotor activity of scrapie-affected mice at late stages of the disease ***(52)***. In complete contrast, Suckling et al. ***(50)*** reported on an "excitable" phase, in which scrapie-affected mice became hyperactive shortly before developing the classic signs of disease at the incubation period, but displayed a reduction in mobility early during the course of the disease. Finally, Savage and Field ***(48)*** did not show any changes in motor activity in scrapie-affected mice. Our results on the passive avoidance differ from previous work done by Hunter et al., who showed that, in a different mouse strain,

there was a mild impairment of learning with the 79A and 139A strains of scrapie, but not with the ME7 or 22C strains ***(52)***. Hunter et al.'s study tested the mice when they were approx 95% of the way through the incubation period, and at a time when the authors state that the activity of the scrapie affected mice was reduced ***(52)***. It is difficult to interpret these results, because the performance of the mice on the passive-avoidance task is likely to be profoundly affected by their reduced activity. Nevertheless, the early behavioral changes we have reported indicate that there is abnormal hippocampal neuronal functioning in prion-affected mice, which may be occurring as a consequence of the inflammatory response in the brain.

5. The Role of Inflammatory Response in Murine Prion Disease

5.1. The Role of Microglia

There is accumulating in vitro evidence that microglia may be activated by PrP^{Sc}, and that these microglia produce potentially neurotoxic molecules. The potential role of microglia in the pathogenesis of the prion diseases, based on these observations, is discussed in detail in Chapter 4. However, it is important to note that studies on microglia in vitro do not necessarily reflect the activities and functions of microglia in vivo. This difference is well illustrated by the contrasting antigen presentation characteristics of microglia in vivo and those of cultured microglia (*see* **ref. *54***). A number of studies have examined the activities of microglia and the inflammatory pathways in prion disease in vivo.

Activated microglia, by virtue of their close spatial association with areas of PrP^{Sc} accumulation, have been postulated to have a role in the production and processing of PrP^{Sc} ***(5,55,56)***. PrPmRNA has been detected in isolated microglia ***(57)***, and PrP^{Sc} immunoreactivity within microglia has been shown in a mouse model of prion disease ***(58)***. Furthermore, Jeffrey et al. ***(59)*** have ultrastructurally localized PrP^{Sc} to the lysosomal compartment within microglia located at the periphery of amyloid plaques. However, although Muhleisen et al. have confirmed that PrP^{Sc} deposition occurs in the same regions as microglial activation in human CJD, they have shown that very few microglial processes are associated with PrP^{Sc} immunoreactivity, and that activated microglia did not contain PrP^{Sc} ***(6)***. Together, these data suggest that microglia, rather than synthesizing PrP^{Sc} *de novo*, may have a role in the phagocytosis of exogenous PrP^{Sc}, which is then processed within their lysosomal compartment. Indeed, there is a substantial body of evidence indicating that PrP^{Sc} processing within the lysosomal compartment may play a central role in the pathogenesis of the prion diseases ***(60–62)***. The microglia may themselves become disease targets, as indicated by the presence of microglia with atypical morphology and occasionally intracytoplasmic vacuolation, and a consequent disturbance

of their normal homeostatic function in the CNS may then contribute to the development of neurodegeneration in the prion diseases ***(21)***.

The mechanisms of neurodegeneration in prion disease remain the subject of investigation, but the most telling clue is that the distribution of neuronal loss is not directly dependent on the deposition of PrP^{Sc} ***(3)***. It seems possible that the contribution of activated microglia may lie in their neurotoxic potential. It is known that microglial activation is accompanied by the release of a number of mediators that have been implicated in causing neurotoxicity, including proteases, cytokines, and free radicals ***(63–65)***. Despite the obvious morphological and immunocytochemical evidence that the microglia are activated in murine prion disease, we have been unable to detect the cytokine interleukin 1 (IL-1β), either immunocytochemically or on enzyme-linked immunosorbent assay, at any point during the course of the disease in ME7-injected mice ***(66)***. Furthermore, at terminal disease, using quantitative competitive reverse transcriptase-polymerase chain reaction, we have been unable to detect any significant difference between ME7- and NBH-injected brains for IL-1β, tumor necrosis factor-α (TNF-α), IL-6, or interferon-γ (IFN-γ) mRNAs. These findings are consistent with a recent study showing "weak and inconstant" IL-1β and TNF-α expression in human CJD ***(3)***, but contradict previous studies based on immunocytochemistry of cytokine expression, both at end-stage disease and during the course of the murine prion disease ***(18,67)***. It is possible that the absence of cytokine expression in our studies is scrapie-agent-strain-related, because the studies that have previously reported cytokine expression used the 22A, 87V, and 301V strains of scrapie. However, given that all of these strains share with ME7 neuronal degeneration and a florid end-stage microglial response ***(4)***, it is surprising to find that only some of them are associated with the production of cytokines by microglia.

The absence of pro-inflammatory cytokines in the presence of a florid microglial response is surprising, given the data that are available on cytokine production by microglia ***(68)***. The profile of cytokine production by microglia has been described using isolated microglia in vitro, which are known not to retain their in vivo phenotype ***(69)***. It certainly appears that cytokines are implicated in the neurotoxicity seen in acute models of CNS injury, such as ischemia, and there is evidence that IL-1β, in particular, exacerbates acute neuronal degeneration ***(70)***. In contrast to either in vitro or in vivo models of acute CNS injury, the murine model of prion disease is characterized by pathology that evolves over many weeks, rather than as an isolated response to a single one-dose stimulus. The acute models of CNS injury cannot mimic the evolving dose–response relationships that must operate in a chronic disease, in which the contribution of pathogenic factors (e.g., abnormal protein or neuronal disturbances) will be both cumulative and synergistic.

The slowly evolving pathology, which provides a continuous signal to impact on the microglia, has parallels with the phenomenon of tachyphylaxis in peripheral tissues. Tachyphylaxis in the periphery is seen as a desensitization, with reduced cellular recruitment to sites already exposed to cytokines *(71)*. The intact brain operates mechanisms to attenuate the acute inflammatory response *(10,11)*. It is possible that, in chronic disease compensatory mechanisms supervene to protect the brain from what is clearly a profound inflammatory response. It is possible that an initial low level of synthesis of cytokines, produced early in the course of the disease, switches off any further cytokine production, but another explanation may lie in an opposing increase in anti-inflammatory cytokines. Recently Baker et al. *(72)* have shown that there is an increase in the expression of transforming growth factor (TGF-β) mRNA in mice and rats infected with CJD, and, in our own model, there are also raised levels of TGF-β (Cunningham and Perry, in preparation). TGF-β is known to have anti-inflammatory actions in the CNS, with associated downregulation of proinflammatory cytokines.

Clearly, cytokines are not the only mediators of inflammation, and other inflammatory pathways, including arachidonic acid metabolites and the complement components, may have a role in the pathogenesis of prion diseases. Walsh et al. *(73)* have shown that there is upregulation of cyclo-oxygenase 2 (COX-2) in activated microglia in prion-affected mice. This is seen at 16 wk in our C57BL6J/ME7 model, and the number of COX-2 positive activated microglia increase with the progress of the disease. The COX-2 positive microglia are particularly numerous in areas of vacuolation, but were also seen in areas where vacuolation is less evident. This increased COX-2 expression is likely to result in the synthesis of prostaglandins and free oxygen radical generation, which may in turn contribute to the pathogenesis of the disease. Prostaglandin $_2$, one of the major prostaglandins released by microglia in culture *(74)*, downregulates microglial function, including the production of pro-inflammatory cytokines *(75)*. This would concur with the absence of pro-inflammatory cytokines observed in this model, and certainly merits further investigation.

5.2. The Role of T-Cells

Our model of murine prion disease is characterized by the accumulation of a subpopulation of predominantly $CD8^+$ T-cells. The presence of these T-cells shows that, despite an intact blood–brain barrier, mononuclear cells can enter the brain parenchyma, and, indeed, Williams et al. *(76)*, using bone marrow chimeras, have shown that some monocytes enter the brain during the course of the disease. The presence of $CD8^+$ T-cells has been described in a number of other neurodegenerative diseases, and also in experimental models of CNS dis-

ease. Infiltration of predominantly $CD8^+$ T-cells has been shown in Alzheimer's disease ***(77)*** and amyotrophic lateral sclerosis ***(78)***. The recruitment of predominantly $CD8^+$ T-cells to an area of damage induced by middle cerebral artery occlusion in the rat has been shown ***(79)***, suggesting that the immune system is also involved in ischemic CNS damage. Partial transection of the spinal cord in the mouse is also associated with an acute recruitment of T-cells to the site of injury ***(80)***. The role of these $CD8^+$ T-cells in the pathogenesis of chronic neurodegenerative diseases, and acute brain and spinal cord injury, remains to be elucidated.

It is likely that the T-cells we have observed are monitoring rather than causing neurodegeneration, because T-cell-depleted mice can develop prion disease ***(81)***. Consistent with this idea, it is interesting to note that, despite the increased expression of major histocompatibility complex class I seen in our model, the T-cells themselves are not positive for IL-2 receptor, IL-1β, or IFN-γ staining, and would appear to be switched off. The unique environment of the brain may mediate the downregulation of recruited T-cells. This phenomenon has been reported in T-cells isolated from the brains of mice with Sindibis virus encephalitis, which showed downregulation of cytokine production ***(82)***.

6. The Significance of Inflammatory Response in Murine Scrapie

The presence of the inflammatory response so early in the course of prion disease, before there is any neuronal loss, indicates that CNS inflammation could contribute to the pathogenesis of the disease. This is highlighted by the spatial coincidence of the inflammatory response with astrocytosis, PrP^{Sc} accumulation, and vacuolation, not only in an intracerebral model of prion disease, but also following intraocular injection of ME7 ***(83)***. There is also indirect evidence for the contribution of CNS inflammation to prion pathogenesis, from the work of Ehresmann et al. ***(84)***, who showed that co-infection of mice with a nonpathogenic adenovirus, which would produce inflammation, accelerated the course of the disease; Outram et al. ***(85,86)*** have reported that treatment of scrapie-affected mice with anti-inflammatory steroids delayed the development of clinical signs.

It appears that the inflammatory response and/or PrP^{Sc} accumulation contribute to the behavioral abnormalities we have detected during the early stages of the disease, and we have suggested that such abnormalities arise as a result of abnormal neuronal functioning (*see* ***refs. 45,87*** and *see* Chapter 11). The early pathological and behavioral findings represent an important advance in the preclinical diagnosis of mouse prion disease, because they are robust markers of ongoing disease at a time when neuronal loss has not yet occurred. There are also striking parallels with the natural history of the human prion diseases. In particular, the reports of early behavioral and psychiatric disturbances consistently seen in variant CJD ***(88)***, but also less frequently reported in the other

types of prion disease ***(89)***, suggest that there is also an abnormality of neuronal functioning in the human prion diseases.

The challenge now is to tease apart the relationships among the inflammatory response, PrP^{Sc} accumulation, behavioral changes, and neuronal loss. This should be done with an eye on other chronic neurodegenerative diseases. In particular, there is now compelling evidence that the inflammatory component in Alzheimer's disease may contribute to the development of cognitive impairment and disease pathology ***(15,16,90–94)***.

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11

The Electroneuropathology of Prion Disease

J. Richard Greene

1. Introduction: What Is Electroneuropathology?

Neuropathological studies can reveal a great deal about the appearance of cells and structures within the nervous system during the course of a disease, but they cannot determine which neurons were working properly when the samples were taken or in the period before death. Neurons may look normal, but are they still able to integrate incoming synaptic information, to generate and propagate action potentials, and lay the foundations of new memories? The answers to these questions are central to the understanding of the relationships between neuropathological abnormalities and particular signs or symptoms. Prion diseases also affect laboratory animals, and, as a consequence, it is possible to study them in great detail, from the presymptomatic to the terminal stages. Of course, mice do not report symptoms, but it is possible to assess their health status by using scoring systems such as those described by Irwin ***(1)***, and behavioral tests can, to some extent, model psychiatric disturbances. The crucial benefit is that the functioning of neurons can be assessed in a pathological and behavioral context. The possibility thereby exists to relate abnormalities in the electrophysiological properties of neurons, or groups of neurons, to particular neuropathological or behavioral changes. We have called this approach "electroneuropathology" (**Fig. 1**) ***(2)***.

Electrophysiological techniques have been used to study the effects of prion disease in whole animals, and in slices of living brain. Experiments in whole animals, including humans, have used electroencephalographic techniques. When applied to rats, they have indicated that electroencephalogram changes occur early in the disease, and have consistent onset times in a wide range of different strains of rats ***(3)***. These changes reflect alterations in the properties

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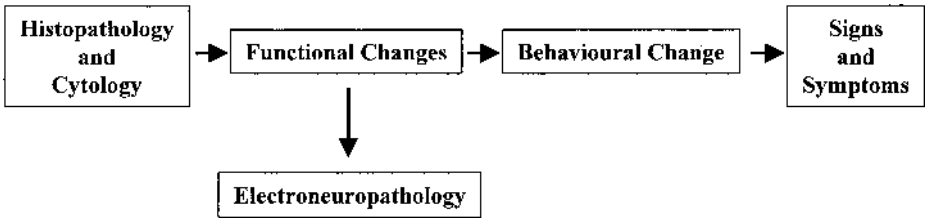

Fig. 1. Electroneuropathology. Application of electrophysiological techniques to study diseased or behaviorally abnormal neuronal tissues.

of groups of neurons, and have been reviewed elsewhere ***(4)***. The majority of studies have used slices of living brain, and it is on these that this chapter concentrates.

2. Experiments Using Slices of Living Brain

Living slices of brain have been used for electrophysiological experiments over many years (**Fig. 2**) ***(5)***. Workers in the prion field have tended to use similar methods for brain-slice preparation and maintenance. Our own methods have been described in detail elsewhere ***(6,7)***, but, briefly, they comprise the following: Under blind conditions, a diseased or matched control mouse is selected, anesthetized, and decapitated. The brain is removed, and horizontal slices of hippocampal formation, 400 μm thick, are then made on a vibroslice (Camden Instruments). Slices are transferred to an interface-type recording chamber, where they are superfused with an artificial cerebrospinal fluid at 36°C. The slices are given 2 h to recover from the preparation procedure, before starting the experiments, and, in general, they remain useful for a further 6 h. The quality of the recordings made in this type of experiment depends on the effectiveness of slice preparation, and this in turn depends on many variables, including the speed and delicacy of dissection, the thickness of slices and method of slicing, and the temperature and composition of the fluid used during dissection and superfusion. Poor technique may result in slices that contain a higher proportion of damaged neurons, which then produce unreliable recordings, and this may be particularly so in diseased tissue.

The same general method can be used to investigate brain tissue of different origins. Experiments have been made on slices from mice with the ME7 strain of scrapie ***(8–12)***, from hamsters with the Sc237 strain ***(13)***, and from transgenic mice with the hamster prion protein gene that have been infected with the Sc237 strain ***(14)***. In general, these studies have examined neurons in the hippocampal formation, but the neocortex ***(13)*** and the lateral geniculate nucleus ***(9)*** have also been studied. The electrophysiological techniques

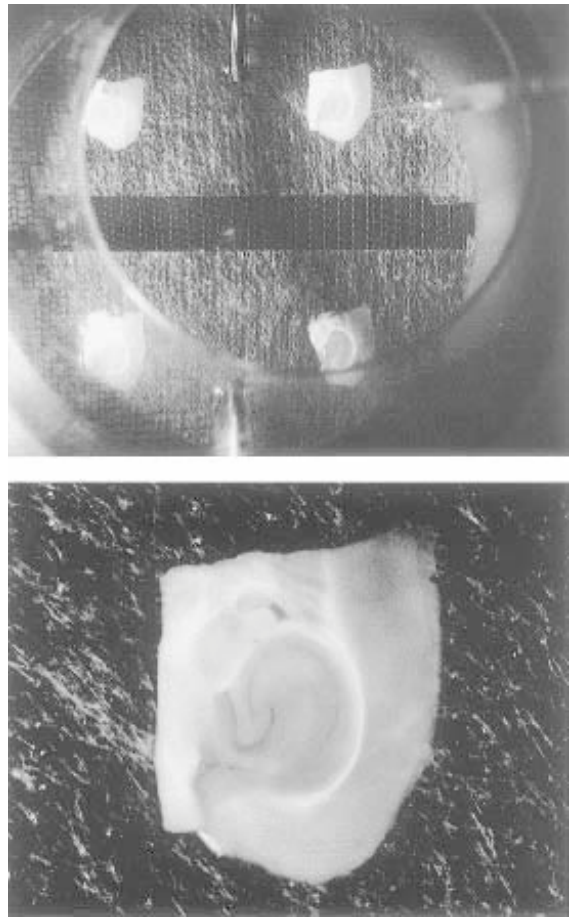

Fig. 2. Brain slice recording chamber. The top panel shows four horizontal slices of mouse hippocampal formation in an interface-type recording chamber. The slices lay on strips of lens-cleaning tissue that are about 1 cm wide, which in turn rest on a mesh of tensioned stocking. They are surrounded by a humidified mixture of 95% oxygen and 5% carbon dioxide. The artificial cerebrospinal fluid (ACSF) enters via the stainless steel tubes seen at the top and the bottom of the picture, and is carried away, by the capillarity of the lens tissue, to an annulus that surrounds the stocking at a lower level. From here, it is sucked from the bath. Flow rates in this chamber are typically 0.1 mL/min (for each side), but are adjusted to ensure that pools of ACSF do not collect around the slice or on the lens tissue. The ACSF is warmed before entering the chamber, which itself sits above a thermostatically controlled water bath. The slices are covered by a lid, which has an aperture to admit the recording (and in some experimental situations, a stimulating) electrode. In this example, the recording electrode is entering at the top right of the picture, and its tip enters the slice in the subiculum. The bottom panel demonstrates that, even in a slice of living, unstained brain 400 μm thick, the subregions of the hippocampal formation can be distinguished easily.

employed have in the main been a mixture of extracellular field potential recording and intracellular current-clamp recording. A central question is whether the symptoms of prion disease are as a result of accumulation of the abnormal protein or a consequence of the loss of functioning of the normal protein. To address this question, experiments have also been conducted in slices from mice that lack the prion protein gene, and therefore presumably lack the activity normally bestowed by the prion protein (assuming that a replacement protein does not compensate) ***(15–20)***. The experiments using null mice have also made wider use of whole-cell and voltage-clamp techniques.

The literature describing the electrophysiological consequences of scrapie infection or prion gene knockout contains contradictions and some confusion. There are valid reasons for differences in results, and these should be borne in mind throughout. They include differences in the species that have been studied, and differences in the strains of scrapie that have been used. The diseases produced by different combinations of host species and scrapie strain have different time-courses and different neuropathological profiles. Of the two chief combinations studied electrophysiologically, ME7 in mice results in marked neuronal loss, and Sc237 infection in hamsters produces no substantial neuronal loss, and has a shorter incubation period.

However, differences in experimental methods are also important. There are undoubtedly subtle differences in the criteria that different researchers use, either consciously or subconsciously, to decide whether or not to include particular neurons in their studies. A brain slice contains millions of neurons, but only a handful will be recorded during any one session. Achieving a representative sample from a control group, which can be compared with another sample that is representative of a scrapie group, is difficult but fundamental. It is necessary to account for the fact that the neuronal population is not homogeneous, and that many neuronal subtypes, with distinct electrophysiological characteristics, exist. It is crucial to compare like with like, so that the only variable between the samples is the presence of disease. In the first instance, all experiments should be done blind to disease status. It is then necessary to keep an open mind about what to expect. By the very nature of the experiments, some of the recorded neurons are sick, and their records will look very abnormal; indeed, they may look like bad recordings from healthy neurons, but these neurons should not be excluded arbitrarily. Acceptance criteria for continuing with a recording should be kept as relaxed as possible, and the setting of minimum acceptable membrane potentials, and so on, should be avoided. The pragmatic view is to accept any neuron in which it is thought likely that the planned experiments can be conducted and interpreted. Finally, when complex and relatively poorly understood phenomena have been investigated, the precise details of the experimental methods employed are likely to have a significant influ-

ence on the results obtained. When such matters of experimental design or conduct are thought to be relevant to discrepancies in the literature, they are highlighted.

3. Effects on Basic Membrane Properties

The basic membrane properties that have been examined are principally the resting membrane potential and the cell input resistance. Studies that have addressed this issue in ME7 mice ***(8,10,11)*** have found the resting membrane potential to be more depolarized (more positive) in neurons from infected mice. In contrast, in Sc237 hamsters, the neurons are not depolarized, compared to controls ***(13)***. When a depolarization has not been found, this may be because depolarized neurons were excluded by the application of predetermined acceptance criteria. Other things being equal, the depolarized neurons would be closer to action potential threshold, and therefore more easily excited. Indeed, increases in the proportion of spontaneously active hippocampal neurons in ME7 mice ***(8)*** and epileptiform activity in transgenic Sc237 mice ***(14)*** have been reported. This change in the pattern of action potential discharge may then lead to accumulation of calcium, activation of enzymes or ion channels, and excitotoxic effects. Although the functioning of high-threshold voltage-gated Ca^{2+} channels has been examined specifically, and found not to be compromised in ME7 mice ***(11)***, this does not exclude the possibility that, in scrapie infection, the resting calcium concentration is elevated.

4. Effects on the Parameters of Action Potential

In ME7 mice, the electrophysiological properties of neurons in area CA1 of the hippocampus have been examined at different, but predominantly later, stages of the disease. The amplitude and the rise time of the action potentials were unaffected, but there was a significant decrease in the fall time ***(8)***, which represents an increase in the rate of repolarization.

The so-called "after-hyperpolarization" (AHP), divided into three components, has also been examined. The early AHP occurs at the end of the repolarization phase of a single action potential (**Fig. 3**); the middle and late AHPs, which are mediated by a range of different voltage- and calcium-activated potassium currents ***(17)***, occur after a sequence of action potentials, and can be difficult to compare accurately among studies. In ME7 scrapie, an increase in the amplitude of the early AHP is reported, and, although not examined in detail, it has been suggested that there is also an increase in the medium AHP ***(8)***.

In area CA1 neurons from Sc237 hamsters, there were also no changes in action potential amplitude or rise time. However, the early AHP was unaffected, rather than increased, and the medium and late AHP were attenuated, rather than increased ***(13)***. These two sets of results are difficult to reconcile.

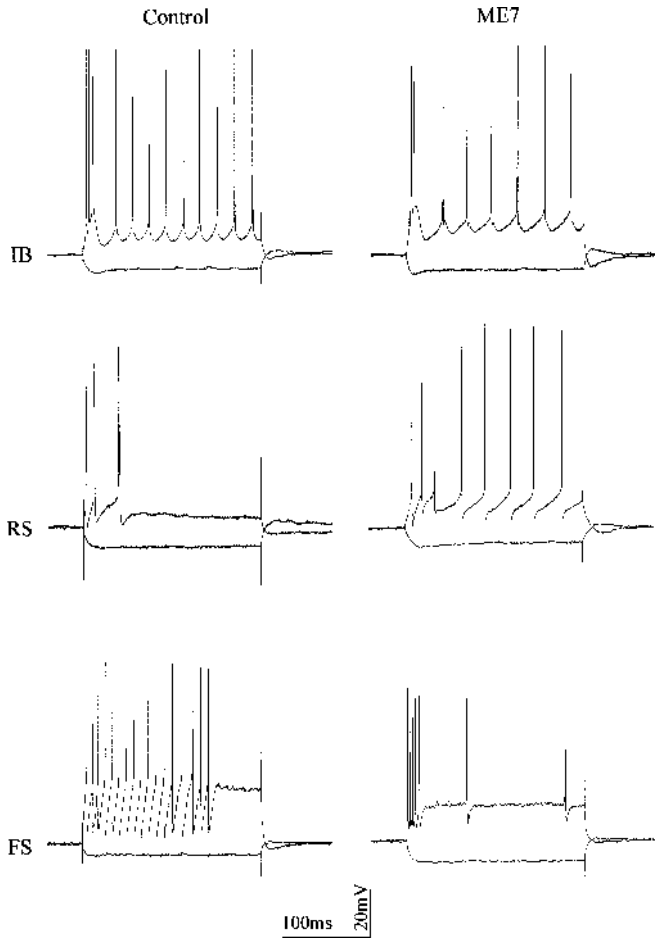

Fig. 3. Types of neuron encountered in control and ME7 mice. Intracellular recordings were made in the subiculum of mice injected, 21 wk previously, with either normal brain homogenate or with ME7 homogenate. The records show, superimposed, the response of each neuron to a depolarizing current pulse (0.4 nA except for the control FS neuron, where it was 0.8 nA) and a hyperpolarizing current pulse (–0.4 nA). Intrinsically burst-firing (IB) and regular-spiking (RS) subtypes of pyramidal neuron are shown. Examples of fast-spiking neurons are given also. It was confirmed morphologically that these were nonpyramidal neurons, but no attempt was made to subtype them. The purpose of this figure is to demonstrate that the same types of neuron can be recorded and distinguished in ME7 as in control mice, and to suggest that failure to account for differences between subtypes could confound a comparison between control and scrapie mice. This figure is not intended to provide a summary of the differences postulated to be present between control and prion affected neurons.

The discordant results for the medium AHPs may reflect differences in the level of analysis, and interpretation should perhaps be left until studies of equal depth have been performed. However, the difference in the early AHP does warrant some consideration.

There is an important philosophical point to consider in this type of experiment, which may be particularly pertinent here: When is a neuron an abnormal example of a particular subclass, and when is it a normal example of a different subclass? The rodent hippocampal formation contains pyramidal neurons that fire single action potentials, these are termed "regular-spiking" (RS) neurons. It also contains neurons that fire bursts of action potentials, riding on single depolarizing waves, and these are termed "intrinsically burst-firing" (IB) neurons *(7,21)*. Examples of the different classes of neurons recorded in control and ME7 mice are given in **Figure 3**.

RS neurons have a more obvious early AHP *(7)*. Thus, if the sample from a scrapie group contained more RS neurons than did a corresponding sample from a control group, the false impression might be gained that the AHP was increased in amplitude in the scrapie group. Indeed, there is some weak evidence, discussed below, to suggest that hippocampal RS neurons may be less susceptible, and thus perhaps more likely to be recorded. Before the issue regarding the amplitude of the early AHP can be settled, further work is needed in ME7 mice that distinguishes clearly between subtypes of neurons.

As well as differences in the shapes of individual action potentials, changes in the patterns of multiple action potential discharges have also been reported. There was an increase in the proportion of spontaneously active neurons in area CA1 neurons in ME7 mice. Also in ME7 mice, there was a reduction in spike frequency adaptation following depolarizing current pulses, and thus a tendency to fire more action potentials when stimulated in this way *(8)*. In the Sc237 hamsters, synaptic stimulation led to double action potential discharges, rather than to the single action potentials seen in controls *(13)*. In both cases, the changes in the pattern of action potential discharge were consistent with a reduction in the AHP.

5. Effects on Synaptic Transmission

5.1. Extracellular Field Potentials

Extracellular field potential recording techniques have been used to record the integrated response of many cells to an artificial stimulus. In the hippocampal formation, recording usually takes place in the stratum radiatum, and follows stimulation of the Schaffer collaterals, some 500 μm away. The amplitude and waveform of the recording are influenced by the number of axons successfully stimulated and the number of neurons contributing to the recorded signal. In Sc237 hamsters, the field potential recorded in the hippocampal formation,

at different stages of the disease, was no different from controls. In contrast, in ME7 mice, there was a marked reduction, and, in the terminal stages of the disease, it was impossible to record ***(8)***. The most likely reason for this discrepancy lies in the pathological consequences of infection in the two cases. In ME7 mice, there is marked cell loss, but this is not the case in Sc237 hamsters. A lower density of neurons may well produce a decrease in the field potential. These differences emphasize the importance of placing the electrophysiological data in their proper pathological context: the essence of electroneuropathology, in fact.

5.2. Intracellular Recordings

Experiments have been conducted in which the recording electrode is placed intracellularly, and the responses of single neurons to spontaneous or evoked synaptic stimulation are assessed. Although the electrode is recording the response of a single neuron, that neuron is in contact with other cells, and should not be regarded as in any way isolated. The only synaptic components that have been examined in any detail in scrapie are the excitatory postsynaptic events mediated by glutamate acting at *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors, and by inhibitory postsynaptic events mediated by γ-aminobutyric acid (GABA) acting at $GABA_A$ and $GABA_B$ receptors. Two forms of recording technique have been employed. In so-called "current clamp," events are recorded as changes in membrane potential, and are referred to as excitatory postsynaptic potential (EPSP) and inhibitory postsynaptic potential (IPSP). In voltage clamp, the membrane potential is kept constant, and events are recorded as changes in membrane current; thus, they are referred to as excitatory or inhibitory postsynaptic currents (EPSC and IPSC, respectively). Differences in experimental technique can be important when comparing apparently disparate sets of results.

It is possible to record a near-normal EPSP in ME7 mice, but only if the stimulus intensity is turned higher than is used in controls ***(12)***. This increase in stimulus intensity is probably needed to compensate for the neuronal loss associated with ME7 in mice. In Sc237 hamsters, in which there is no marked neuronal loss, normal EPSPs were recorded without any need to increase the stimulus strength ***(13)***. These results imply that any change in the EPSP results from a change in the density of neurons being stimulated, rather than from a specific effect of prion infection on the postsynaptic machinery that underpins the EPSP. There is less agreement about IPSPs. Although not studied in detail in ME7 mice, evoked IPSPs appeared to be attenuated ***(8)***. On the other hand, in Sc237 hamsters, the $GABA_A$-mediated IPSP, assessed using multiple criteria, was unchanged ***(13)***. More detailed studies of the IPSP in ME7 mice are needed to resolve this issue.

5.3. Long-Term Potentiation

Long-term potentiation (LTP) is the phenomenon, first described in detail by Bliss and Lomo ***(22)***, in which repeated use of a synapse strengthens the functional connection across that synapse. The reasons why those studying a dementing illness are drawn to a phenomenon described as a synaptic model of memory ***(23)*** are obvious. In the experimental setting, synaptic potentiation that persists for more than 1 h is usually regarded as LTP, but there are other forms of synaptic potentiation that are less long-lived. In ME7 mice at the later stages of the disease, but before there is frank neuropathological change or loss of dendritic spines, the ability to maintain LTP is lost. In these mice, although the stimulus protocol failed to produce LTP, it continued to produce short-term potentiation ***(12)***. These results have been interpreted as a PrP^{Sc}-induced loss of the ability to change short-term potentiation into LTP. The locus of this effect is likely to be postsynaptic, but occurs before there are detectable changes in the numbers of dendritic spines ***(12)***.

6. Electrophysiological Changes in Prion-Null Mice

It is important to know if mice that lack the prion protein gene are electrophysiologically normal or not. If they are, this suggests that loss of the functioning of the normal prion protein is not of great importance, and that the symptoms of prion disease stem from the presence of the abnormal protein. Abnormal knockouts suggest that loss of the functioning of the normal protein is important. Furthermore, any such abnormalities, similar to those seen in animals with prion disease, support the view that loss of the normal is important, rather than accumulation of the abnormal. It is prudent to keep in mind the caveat that the function of the missing proteins may have been taken over by different proteins. Unfortunately, electrophysiological experiments using knockout mice have yielded a range of different results. The passive membrane properties of null mice are unchanged ***(15,19)***, and this is at odds with the results obtained in ME7 mice ***(8,10)***, but in agreement with those from Sc237 hamsters ***(13)***. The medium AHP is reduced in Sc237 hamsters ***(13)***, but not in null mice ***(17)***. However, the late AHP is reduced both in null mice ***(17)*** and in Sc237 hamsters ***(13)***, and this suggests that prion protein is in some way involved in the calcium-activated potassium current that mediates the late AHP ***(17)***. Thus, for passive membrane properties, and for the late AHP, there is concordance between the data derived from prion-null mice and Sc237 hamsters, but not between prion-null mice and ME7 mice.

In prion-null mice, attenuation of evoked, $GABA_A$-mediated IPSCs has been reported ***(15)***, but not replicated ***(19)***. Synaptic transmission has also been investigated using somewhat different techniques in thin slices (150 μm, rather than the 400 μm used in other experiments) of cerebellum ***(18)***. In these experi-

ments, spontaneous, rather than evoked, IPSCs were measured and found to be normal. Some degree of caution is required when comparing these two results, because they represent different phenomena. A spontaneous IPSC is likely to be the result of a discharge from a single inhibitory neuron; an evoked IPSP may result from several neurons. It has also been argued that, because spontaneous IPSCs are small, they would have been difficult to measure accurately ***(13)***. On the other hand, inhibitory events mediated by $GABA_A$ receptors may be measured more accurately as currents, using voltage-clamp techniques, because very small changes in the resting membrane potential can have profound effects on the amplitude of the IPSP.

In these same experiments, the EPSC, elicited in cerebellar neurons by stimulation of the climbing fibers, was also investigated and found to be normal ***(18)***, and this result does agree with previous studies that have reported the EPSC to be qualitatively normal ***(15)***. Thus, there is agreement that the EPSC is unchanged in both infected and knockout animals. However, regarding the IPSPs, although there is agreement among some studies derived from prion-null mice and Sc237 hamsters, data from prion-null mice and ME7 mice do not agree.

Investigations of synaptic plasticity in relation to prion disease are particularly fraught with mistakes. LTP was reported to be abnormal in the null mice ***(15)***. A letter describing a replication of this finding was published the following year by a different group ***(16)***. However, a subsequent study by a leading LTP laboratory failed to find any differences in LTP between prion-null and control mice, under blind conditions ***(19)***. It was suggested at the time that this discrepancy resulted from differences in the genetic backgrounds of the knockouts. This seems unlikely, given that LTP was apparently normal in knockouts with three different backgrounds ***(19)***. Thus, on balance, LTP is probably normal in prion-null mice.

The difficulty of using LTP to probe the functions of the prion protein lies primarily in the lack of consensus about the mechanisms that underlie LTP. It is generally agreed that a rise in the postsynaptic calcium concentration is necessary, and occurs as a consequence of calcium entry through NMDA receptor channels that open in response to membrane depolarization ***(23)***, but there has been much debate surrounding the primary locus of LTP. If the locus is at least in part presynaptic ***(23)***, this would point to a role for prion protein in transmitter release, and this possibility is supported by the results of experiments in cerebellar Purkinje cells ***(18)***. If, as much current thinking favors ***(24)***, the locus is predominantly postsynaptic and involves the unmasking of silent synapses by the translocation of glutamate receptors it is possible that prion protein has a role in the membrane trafficking of such receptors.

Ironically, just as attention is focused on a postsynaptic locus for LTP, recent results suggest that normal prion protein is concentrated presynaptically ***(25)***.

A presynaptic effect of PrP is also suggested by the results of a small number of experiments in cerebellar Purkinje cells. It has been reported that the sum amplitude of spontaneous IPSCs was reduced by the application of copper ions in prion-null, but not in wild-type mice ***(20)***.The LTP literature also counsels on the difficulty of making valid comparisons between sets of results, when different methodologies have been used to obtain them ***(26)***. Factors that are likely to affect results, not only of the LTP experiments, but of many of the experiments described in this chapter, include the age of the animals, the brain region, the presence (or not) of intact synaptic inhibition, the details of the recording technique, the temperature, composition of bathing and electrode filling solutions, and the measurement of EPSP amplitude, rather than slope. This does not mean that all conflicting results are equally reliable and have valid explanations. As far as prion proteins and LTP are concerned, it is too soon to tell.

Thus, overall, the electrophysiological studies have not yet establish conclusively whether it is loss of normal protein or accumulation of abnormal protein that is important to the generation of signs of disease.

7. Differential Vulnerability Among Neuronal Subtypes

Whether or not some neurons are more vulnerable to the effects of PrP^{Sc}, be they direct or indirect, is an important question. If the answer is yes, and the vulnerability resides in a pharmacologically accessible aspect of the neuron, it may be possible to design drugs that remove that vulnerability. In our studies on ME7 mouse subiculum, no evidence was found of a differential loss of nonpyramidal neurons (note that nonpyramidal neurons were not subtyped), or indeed a differential loss of one subtype of pyramidal neuron over another **(Fig. 4)**.

In the neocortex, differences in the action potential in Sc237 mice are present in RS, but not IB neurons ***(13)***. In ME7 mice, even at late stage, some electrophysiological and morphologically normal lateral geniculate neurons remain ***(9)***. In studies in the subiculum, membrane depolarization was present in IB, but not RS, neurons ***(10)***. Thus, the electrophysiological data suggest that particular neurons are more vulnerable than others. The explanations for this are more elusive, but could lie in the anatomy of neuronal circuits. It has been suggested that, in subiculum, IB and RS neurons receive different inputs, and have different projection targets ***(7,27)***. It may simply be that the abnormal protein reaches IB neurons more quickly, because of differences in the length of circuits or in the numbers of interposed synapses. However, in the rat subiculum, IB and RS neurons do have different pharmacological properties, and these could relate to vulnerability. RS neurons contain the neuronal form of nitric oxide synthase, but IB cells do not ***(28)***. IB neurons respond much more

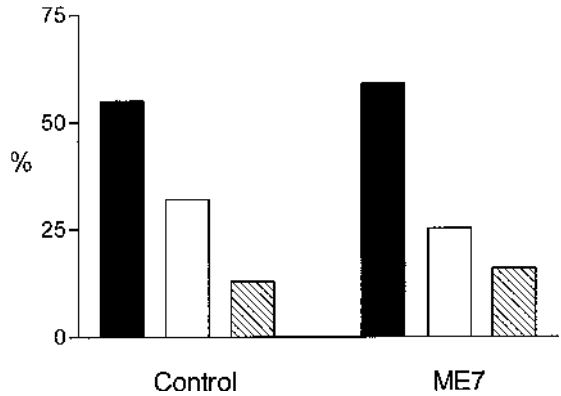

Fig. 4. Proportion of neuronal subtypes recorded in control and ME7 mice. Recordings were made in the subiculum: 22 neurons were recorded from three control mice that had received intrahippocampal injections of normal brain homogenate 21 wk previously, and 32 neurons from four matched mice that had been injected with ME7 homogenate. Solid bars indicate the proportion of neurons that were of IB pyramidal subtype, open bars, the RS pyramidal subtype; and the hatched bars, the proportion of interneurons.

strongly to the neuropeptide, somatostatin ***(6)***. IB neurons also show a much more prominent sag in the voltage response to hyperpolarizing current pulses ***(21)***, and this is known to be true also in guinea pig ***(29)*** and mice ***(10)***. This sag is thought to be mediated by a mixed cation current, referred to as I_h . Potassium is one of the charge carriers involved in I_h ***(30)***, and possible selectivity for neurons that express much sag adds support to the view ***(11)*** that potassium currents are involved in scrapie infection.

8. Abnormalities of Neuronal Morphology

A number of studies have been made in which the electrophysiologically characterized neurons have also been filled with an intracellular label, such as Neurobiotin (Vector), and examined morphologically. These studies have revealed a spectrum of abnormalities. In a small study in ME7 mice, there was a loss of dendritic spines and the presence of a small number of membrane diverticuli ***(8)***. We found more severe morphological abnormalities in ME7 mice ***(10)***. This may be because very relaxed acceptance criteria were used, and we may therefore have sampled from more severely affected neurons. Neurons were found where only the apical dendrite remained, and branching was very much less extensive. Neurons were also found in which the label has apparently been excluded from some regions of the soma and apical dendrite; An example of this is given in **Figure 5**. The localization and the scale of these filling defects suggests that they are caused by the presence of (unlabeled)

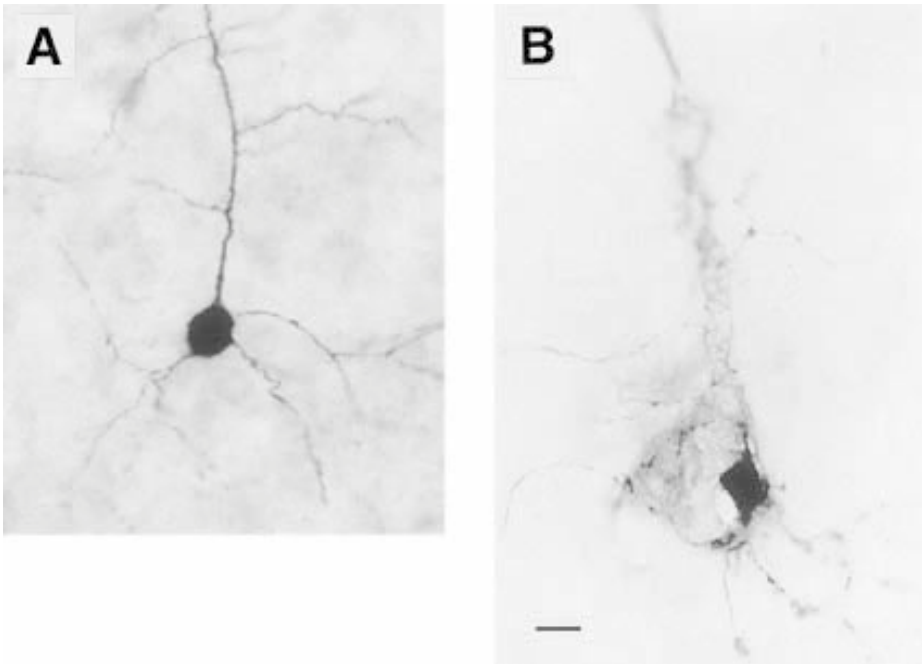

Fig. 5. Morphological changes in neurons from ME7 mice. Two subicular pyramidal neurons (IB subtype), that have been filled with Neurobiotin during electrophysiological characterization, are shown. **(A)** is from a control mouse, and has the typical appearance of a pyramidal (projection) neuron. There is flask-shaped soma with prominent apical dendrite, a skirt of basal dendrites, and the presence of some dendritic spines, note that the intracellular dye is evenly distributed. **(B)** This neuron is from a scrapie-infected mouse, note that the neuron is distended, and that the intracellular dye is displaced from some regions of the soma and the apical dendrite, giving a honeycombed appearance. The scale bar is 10 μm.

coalescences of mitochondria or lysosomes, which have been reported to occur in scrapie infection ***(31,32)***. In general, the morphological abnormalities found in electrophysiologically characterized neurons from ME7 mice are similar to those reported using more traditional techniques. Golgi impregnation and confocal microscopy in ME7 mice reveals that the first morphological abnormality is a loss of dendritic spines ***(33)***, and that swelling of the dendrites, intraneuronal vacuolation, and extensive neuronal loss follow.

In contrast, in a detailed study of electrophysiologically characterized neurons from terminal-stage Sc237 hamsters, there were only mild morphological changes, consisting of an increase in the arborization of the basal dendrites, and no change in the numbers of dendritic spines ***(13)***. This may be a sampling issue, but it may also be that those neurons with severe morphological abnor-

malities are those that are destined to be lost, and these would be expected to be of greater number in ME7 mice, in which neuronal loss is a feature, than in Sc237 hamsters, in which it is not.

9. Conclusions and Future Directions

It is clear that the electrophysiological effects seen in prion-affected mice are sensitive to the combination of scrapie strain and animal species employed. Different combinations produce distinct neuropathological changes, and it is likely that the underlying pathology determines the electrophysiological changes that are seen. Electrophysiological studies point to changes in potassium currents and alterations in intracellular calcium concentrations. There is also evidence to suggest that the locus of effect may be both pre- and postsynaptic. At present, it appears that not all neurons are affected equally, but the basis of any difference in vulnerability is not understood. Nor is it possible to say with confidence if the electrophysiological effects seen result from the direct effects of PrP^{Sc}, to a loss of functioning of PrP, or indeed to mediators released by the host in response to the presence of PrP^{Sc}. Previously, prion infection has been shown to be associated with an inflammatory response ***(33–37)***. It is known that some mediators of inflammation in the brain have their own electrophysiological effects ***(38,39)***, and it is possible that these contribute to the overall picture seen in acute slices from diseased animals. Experiments are needed in which the effects of the normal and the abnormal forms of the prion protein are assessed by applying them directly to neurons in an experimental environment that excludes the complication of an inflammatory response.

At first sight, the contradictions in the literature are annoying: Some may result from differences in methodology, but others may have a solid biological basis. After intracerebral inoculation, both ME7 mice and Sc237 hamsters develop gross neurological symptoms and die from their disease in a predictable fashion. However, in these two models, the neuropathological characteristics are different. In ME7 mice, there is marked neuronal loss; in Sc237 hamsters, neuronal loss and neuropathology are much less apparent (at least in the brain regions that have been studied), but the hamsters die anyway ***(13)***. One interpretation of these results is that, in the Sc237 hamsters, electrophysiological changes (and signs of disease) are caused directly by alterations in the prion protein whereas in the ME7 mice, in addition to these effects, there are electrophysiological abnormalities related directly to the neuropathological changes that occur. In this respect, the ME7 model may be closer to the human condition. If death really can occur without extensive neuropathological involvement, this has implications for identifying potential therapeutic targets. These issues must be clarified, and this can be achieved by the application of the electroneuropathological approach, in which electrophysiological changes

are placed in the context of the pathological status of the individually characterized neurons themselves.

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12

Transmissible Spongiform Encephalopathy Neurobiology and Ultrastructure Suggests Extracellular PrP^{Sc} Conversion Consistent with Classical Amyloidosis

Martin Jeffrey and Jan R. Fraser

1. Introduction

The infectious cause of the transmissible spongiform encephalopathies (TSEs), or prion diseases, is not yet clearly defined. Although minorities of researchers cling tenaciously to the virus hypothesis, the prion or protein-only hypothesis is now widely accepted by most scientists working in this field ***(1)***. When originally formulated, the protein-only hypothesis proposed that the TSE agent was an abnormal infectious form of a host-coded protein (protease-resistant protein, or prion protein) that could convert homologous normal forms of the protein into replicates of itself ***(2)***.

It has become clear that the infectious agent is unlikely to be this abnormal form of the prion protein acting on its own. A subsequent lack of clarity about what constitutes a prion has led to a number of different notations (PrP^{Sc}, PrP^{*}, PrP^{res}) being used to refer either to the infectious particle or the abnormal isoform of PrP, or to both. The principal feature most commonly used to distinguish between the disease-associated forms of PrP and the normal isoform is the relative protease resistance of the abnormal form, compared to the normal PrP isoform. This chapter uses the nomenclature of Caughey and Chesebro, in which PrP^{res} is the abnormal form of the PrP and PrP^{sen} is the normal form ***(3)***. PrP^{res} is not a prion, which is the word invented to describe the hypothetical agent consisting solely of protein ***(1,2)***.

None of the antibodies currently available for routine use in immunocytochemistry is able to reliably distinguish between PrP^{sen} and PrP^{res}, but it is

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frequently stated that pretreatment of sections with formic acid destroys PrP^{sen} ***(4,5)***. However, this has not been unequivocally validated, and it is our experience that at least some forms of PrP^{sen} do survive formic acid pretreatment. Because immunocytochemistry alone is unable to determine whether the PrP molecule is in an abnormal or a normal isoform, we use the term "disease-associated PrP accumulation," which is without prejudice as to the protease sensitivity of the abnormal deposits. The advantage of immunocytochemistry compared to other detection methods for PrP, is that it can detect abnormal accumulations of PrP in relation to individual cells, not only at light microscopy but also at subcellular levels.

This chapter records, in particular, the ultrastructural similarities between PrP amyloid formation and that of other conventional noninfectious amyloidoses. As the prion hypothesis has become increasingly accepted, it has become difficult to make mention of other possible causal agents and retain credibility. Nevertheless, we will also briefly comment on a poorly understood group of structures, the so-called tubulovesicular bodies, which are within the appropriate size range, and have some morphological similarity to conventional viruses, and have still not been molecularly characterized.

2. PrP Aggregation and Fibrillization

One of the hallmark features of the pathology of TSEs is the formation of amyloid plaques ***(6–8)***, which are numerous in some murine models and in some forms of the human TSEs, most notably kuru and variant Creutzfeldt-Jakob disease (vCJD). The sites and deposition, form and structure of the amyloid plaques is under host and agent control ***(7,9)***. Most plaques are formed in association with neurons, but cerebrovascular plaques, probably resulting from infection of endothelial cells ***(10)*** form in natural sheep scrapie and in the 145Y stop mutation of human Gerstmann-Sträussler-Scheinker syndrome (GSS) ***(11)***, and in a murine chronic-wasting disease model. Subependymal and subpial plaques may be commonly found in some murine models. However, plaques are not found in some TSE diseases, such as in bovine spongiform encephalopathy (BSE) ***(12,13)***. Ultrastructure shows that classic plaques are composed of a stellate array of bundles of amyloid fibrils surrounded by reactive astrocytes and microglia ***(14–16)***. It is these bundles of amyloid fibrils that result in the typical staining patterns of amyloid, such as congophilia and birefringence, under polarized light. Immunogold staining has confirmed that the amyloid present in these plaques is composed mostly of disease-specific PrP (**Fig 1**; ***14,15***).

Amyloid plaques are initially formed at focal points along the length of dendrites, and possibly along axons at nodes of Ranvier. These plaques, which we call "primitive plaques," are visible as round or oval areas of immunostaining, when examined by light microscopy but they do not show conventional tinctorial

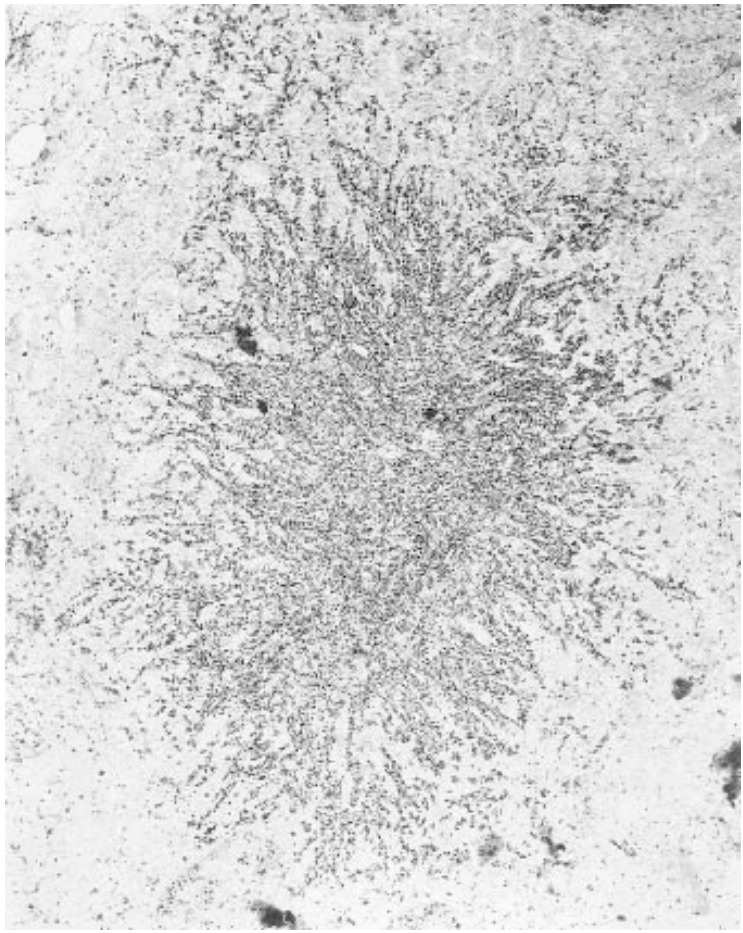

Fig. 1. Electron microscope micrograph of a classic or kuru-type plaque located in the cerebral cortex of an 87V scrapie-infected mouse. The preparation has been stained using an immunogold silver method, which reacts with PrP. The plaque shows the characteristic stellate appearance formed by radiating bundles of amyloid fibrils, which are decorated with immunogold silver reaction product, indicating that the fibrils are composed of PrP.

staining patterns of amyloid ***(15,16)***. Multiple plaques are often formed along the length of the primary dendrite of cerebrocortical and hippocampal pyramidal neurons. Ultrastructurally, these primitive plaques show accumulation of PrP at the plasmalemma of dendrites ***(15)***. This PrP is not in a visibly aggregated form (preamyloid), and diffuses away from the point of release, spreading through the extracellular space to surround other neurites and glia of the adjacent neuropil ***(15–17)***.

Although the electron microscope does not allow resolution of individual molecules, or even of small multimers of PrP, soluble forms of PrP have been found in tissue culture and in the cerebrospinal fluid of CJD patients ***(18)***. It is therefore likely that the diffusable preamyloid PrP, revealed by immunogold reaction in the electron microscope is in a soluble form. In many neuroanatomical areas, the preamyloid PrP initially released into the extracellular space does not at first result in morphological evidence of pathology, either by light or electron microscopy. It does not elicit an astroglial or microglial response, and is not found in association with neuritic pathology, neuroaxonal dystrophy, or neuronal loss. (Similarly, in spleen, PrP is initially released without evident changes in follicular dendritic cells ***[19]***).

Extensive zones of diffusion around dendrites, in the absence of aggregation, suggest that the preamyloid PrP release and diffusion may occur over a long period of time. The formation of individual fibrils occurs within the extracellular space often at some distance from the releasing cell or dendrite ***(16,20,21)*** . Once fibrillar amyloid becomes visible, there appears to be a rapid acceleration of fibril formation, and single fibrils are found throughout the zone of extracellular PrP accumulation ***(15,16,21)***. The appearance of both aggregated and fibrillar PrP is associated with a increased astroglial and microglial response ***(21)***. In addition, marked fibrillization coincides with evidence of increased receptor-mediated endocytosis, as evidenced by increased coated (presumed clathrin-coated) pit activity. Immunoreaction to PrP may be seen in some of these coated pits ***(17,21)***. These features are also seen in association with fibrillization of PrP around follicular dendritic cells of the spleen (***19***; **Fig. 2**). The increased clathrin-coated activity is not seen for preamyloid PrP accumulations. These findings suggest that abnormal forms or aggregates of PrP bind to receptors at the cell surface of neurons, glia, follicular dendritic cells and lymphocytes.

Because none of the antibodies currently available for immunocytochemistry is able to discriminate between normal host-coded PrP^{sen} and PrP^{res}, it is not clear whether the preamyloid forms of PrP or the fibrillar forms of aggregated PrP contain PrP^{res}. However, it is well established that proteins that form amyloid fibrils contain a high proportion of β-pleated sheet, and it is therefore reasonable to assume that the PrP forming amyloid fibrils is abnormally configured, relative to PrP^{sen}, and contains a high proportion of β-pleated sheet. The PrP^{res} detected by immunoblotting are N-terminally truncated near the octarepeat segment of the normal protein ***(22)***. However, both the preamyloid forms of PrP and the PrP that forms the amyloid plaques of murine scrapie and cerebrovascular amyloid of sheep contain the N-terminus of the PrP molecule ***(10,23)***. The intensity of the immunoreaction with the N-terminal antibodies indicates that both preamyloid PrP and fibrillar PrP are composed mostly or

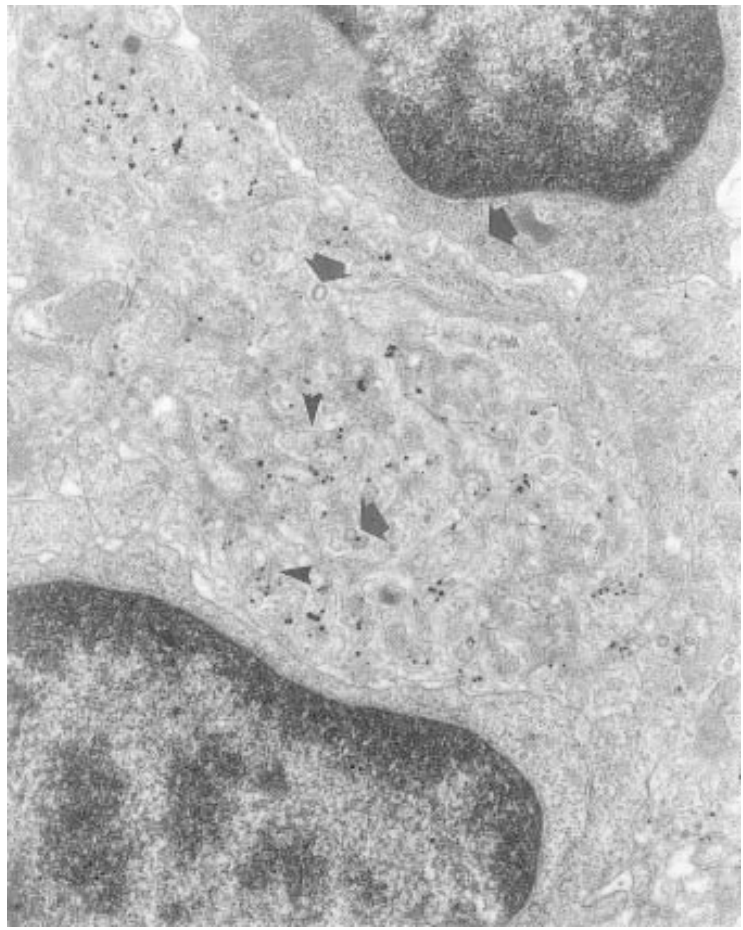

Fig. 2. Scrapie-infected, highly reactive cytoplasmic processes (dendrites) of a follicular dendritic cell from the spleen of an ME7-infected mouse (immunogold silver method for PrP). The deposition of the immunogold reaction indicates that there is marked accumulation of PrP in the extracellular space around the cytoplasmic processes of the infected cell. Amyloid fibrils (arrowheads) are also present in the extracellular space, and both the dendrites of the follicular dendritic cell and adjacent lymphocytes show numerous structures morphologically identified as clathrin coated pits (arrows).

entirely of the whole-length form of the protein. The relationship between the N-terminally truncated PrP^{res} and immunodetected PrP is therefore unclear, but it is likely that the acquisition of protease resistance follows aggregation of the protein and that most N-terminal truncation of disease associated PrP occurs after biochemical treatment of the extracted protein. It is therefore possible that the PrP released into the extracellular space, which does not damage tis-

sue, is in a protease sensitive form of the protein, and it is the local conditions of excess concentration of PrP^{sen} that induce aggregation, fibrillization, and acquisition of protease resistance.

Two models have been proposed to explain the conversion of PrP^{sen} to PrP^{res}: the heterodimer model, and seeded polymerization (*see* ***ref. 24*** for review). It has previously been suggested ***(25)*** that PrP^{res} may convert to PrP^{sen} via a heterodimer molecule. However, previous studies ***(26,27)*** have shown that the sequential dilution of PrP^{res}, when combined with radio-labeled PrP^{sen}, results in a complete elimination of the converting activity. This suggests that PrP^{res} is an ordered oligomeric seed (i.e., a particle many times larger than a monomer or a dimer).

A heterodimeric conversion of PrP^{sen} to PrP^{res} is not dependent on concentration of reagents, and would take place either within the cell or at the cell surface. In contrast, seeded polymerization will occur as the concentration and kinetics of PrP^{sen} to PrP^{res} conversion reach a critical concentration, when the amounts of disease-specific PrP reach the appropriate concentration. That is, in typical nucleation-dependent polymerization of amyloid, polymer is not observed until the monomer concentration exceeds a certain level (the critical concentration) ***(24)***. That diffusion of a preamyloid PrP is observed followed by fibril formation, then rapid generalized formation of fibrils and mature amyloid, is consistent with the seeded polymerization mechanism of classic amyloid, and inconsistent with the heterodimeric model.

In Alzheimer's disease, amyloid also forms within the neuroparenchyma, where Aβ amyloid is dependent on pH. Sedimentable aggregates of Aβ 1–40, grown at pH 5.8, have different properties from aggregates grown at pH 7.4. This feature is reminiscent of the different properties of PrP obtained from different strains of scrapie ***(28)***. The situation in plaques found in GSS is different than that of scrapie, in that the PrP at the center of plaques is N-terminally truncated ***(29–31)***. The proteins forming plaques in GSS patients may be different from the PrP found in normal hosts, and there may therefore be differences in PrP processing in inherited and infectious forms of the TSE diseases ***(32,33)***. PrP molecules, containing mutations associated with familial TSEs, exhibit altered metabolism, and, when expressed in some uninfected tissue culture cells, may show increased aggregation and protease resistance reminiscent of PrP^{res} ***(34,35)***. Even nonmutated hamster PrP^{sen} shows a tendency to aggregate, when expressed as a dimer ***(36)***.

3. Patterns of PrP Accumulation and Cellular Tropism of Scrapie

It has long been known that different patterns of neuropil vacuolation have been found in different sheep breeds and in mice ***(37,38)***. The pattern of vacuolation (lesion profile), in highly inbred stains of mice infected with limiting-

dilution, cloned agent strains, produces highly reproducible selective targeting of lesions. The lesion profile, along with incubation period (determined in groups of S7 and P7 mouse strains), has been used to characterize agent strains ***(39)***. The neuroanatomical pattern of vacuolation is mirrored by highly distinctive patterns of PrP accumulation ***(38,40)***. The nature of PrP staining seen within different neuroanatomical areas of the brain is also distinctive ***(16,17)***. PrP accumulation is most commonly found as localized, diffuse, finely punctate staining. In addition, perineuronal and intraneuronal, periglial, subpial, subependymal, and localized diffuse granular patterns of PrP staining may also be seen in sheep, cattle, cats, and mice and as discussed in **Subheading 2.**, as plaques.

Perineuronal patterns of PrP accumulation are found in natural sheep scrapie, in BSE, in feline spongiform encephalopathy (FSE), and in some murine models. Such patterns are found in many neuroanatomical nuclei, commonly in the reticular formation, in cattle and sheep. In 87V murine scrapie, perineuronal patterns of PrP accumulation are found around neurons of the lateral hypothalamus. The perineuronal pattern is often not confined to the perikaryonal cytoplasm, but may extend to delineate dendrites and axons. Ultrastructurally, PrP is found around the cell, in association with the plasmalemma and adjacent extracellular space of the neuron. As described above, the cells that release PrP into the neuropil are not morphologically abnormal, but can be electrophysiologically normal ***(41)***.

The PrP released from scrapie-infected neurons accumulates in the surrounding neuropil, where it is found in the extracellular space around cellular processes ***(15,17,21)***. The processes include neurites, glial cells, and myelin sheaths. The PrP that accumulates is not visibly aggregated, and, in many neuroanatomical areas, does not cause tissue changes (**Fig. 3**). However, following some unknown stimulus, extracellular disease-associated PrP accumulations form visible electron-dense aggregates within the extracellular space. Following initial aggregation, the PrP then forms individual fibrils. Initially, these are organized haphazardly, but eventually small bundles of amyloid fibrils are seen. Once aggregation starts, fibrillization appears to follow quickly, and sites that initially had only preamyloid PrP such as the immediate perikaryonal neuropil, will form fibrils ***(15,21)***. Once aggregation within the extracellular space begins, there is a marked glial response, with increased numbers of astrocytic processes containing abundant intermediate filament bundles. Microglial activation is also prominent. Aggregated and fibrillar PrP binds other extracellular matrix compounds, such as highly sulphated glycosaminoglycans ***(42)***. As was also seen in plaque formation, these features suggest that aggregated and fibrillar forms of PrP are damaging to the neuropil, although the preamyloid forms of PrP are not invariably damaging.

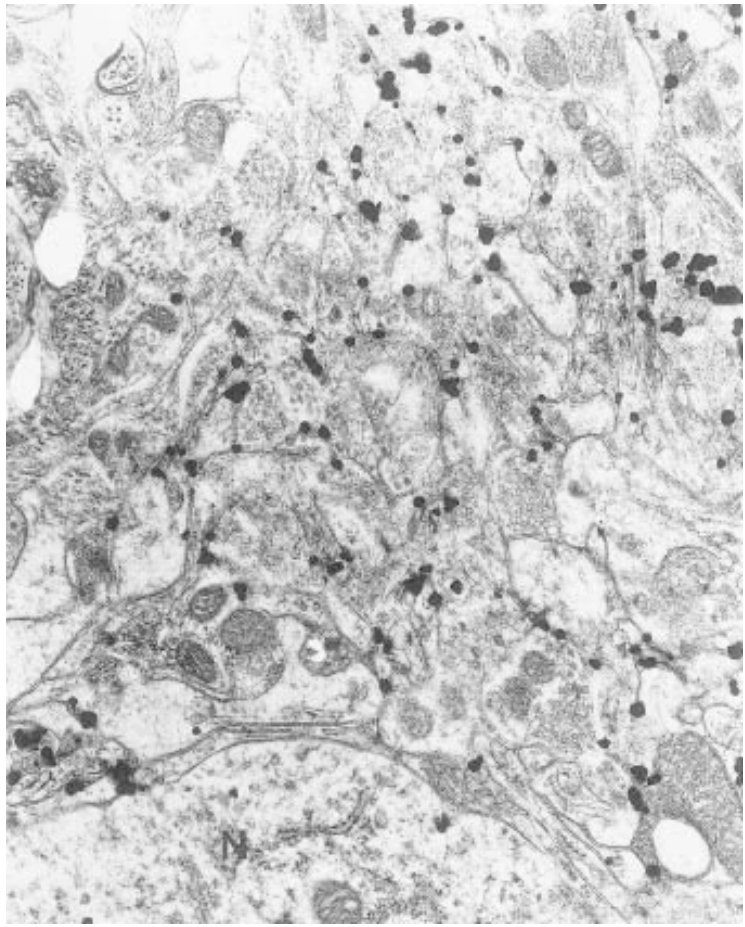

Fig. 3. A scrapie-infected neuron (N) and adjacent neuropil from the lateral hypothalamus of an 87V scrapie-infected mouse (immunogold silver method for PrP). There is marked PrP accumulation within the neuropil. The immunogold reaction is associated with opposing plasmalemmae of contiguous processes (mostly axon terminals and dendrites), suggesting a location to the extracellular space. (The 10 nm immunogold method provides more accurate localization of reaction product, and confirms this distribution). There is no morphologically discernible alteration to the structure of the neuropil associated with this disease-specific PrP accumulation.

PrP accumulation, in association with other cell types in the appropriate disease model (diffuse, periglial and periependymal patterns), appears to follow a similar sequence of events, i.e., PrP accumulation begins initially as release of PrP from the plasmalemma into the extracellular space. PrP accumulates around

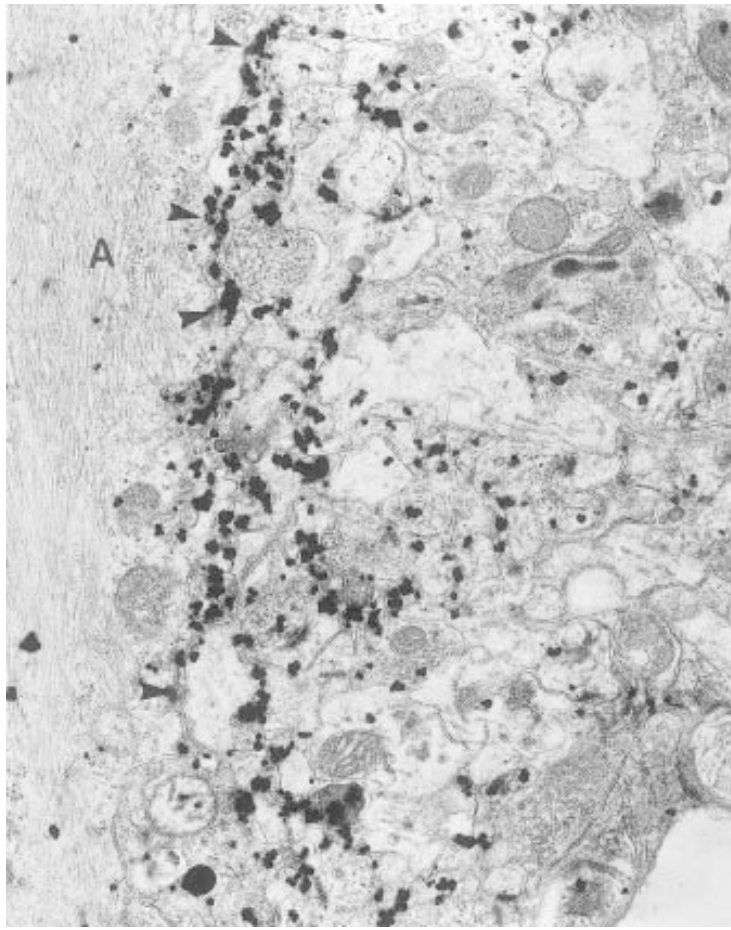

Fig. 4. Part of a reactive astrocyte perikaryon **(A)** adjacent to the corpus callosum of a 301V scrapie-infected mouse (immunogold silver method for PrP). The astrocyte cytoplasm has abundant intermediate filaments. Preamyloid PrP accumulation, revealed by the immunogold reaction deposit, is present at the plasmalemma of the astrocyte (arrowheads). PrP accumulation is also present in the adjacent neuropil, suggesting release and diffusion of disease-specific PrP by the infected astrocyte.

the presumptive infected cells, only subsequently becoming aggregated and forming fibrils, which indicates that several cell types within the central nervous system, including astroglia (**Fig. 4**), ependymal cells, and neurons, may become infected with scrapie. Although we have not studied the subcellular patterns of PrP accumulation for perivascular plaques, in sheep these plaques begin as endothelial cell infections. Subpial and perivascular patterns of PrP accumulation in

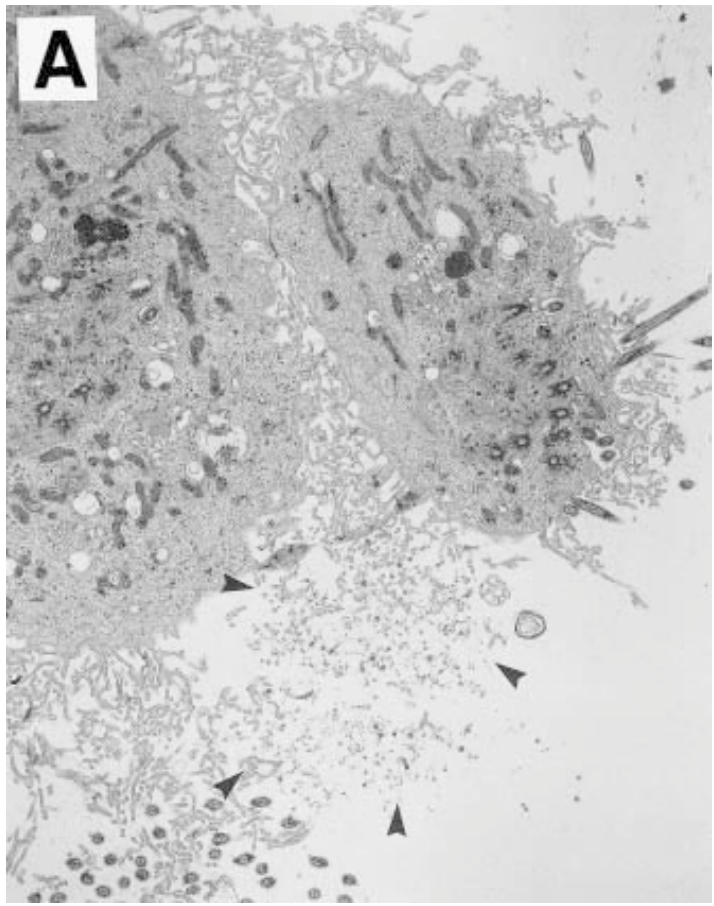

Fig. 5. **(A)** The ventricular borders of two ependymal cells from a 237K infected hamster are shown. There is a focal area of fibril accumulation (immunolabeling not shown), indicated by arrowheads, within the ventricular space. Stained with uranyl acetate and lead citrate.

sheep are a special case of astrocytic infection, as PrP accumulates in association with the glial limitans. Within a single infected sheep brain, areas representative of neuronal, astroglial, endothelial, and ependymal cellular infections may be detected. Although neuronal, glial, and ependymal infection may occur following transynaptic spread, or simply following bulk flow through the central nervous system, endothelial infection must be acquired by hematogenous infection.

In the case of diffuse patterns of PrP accumulation, such as occurs for example in the hippocampus of the ME7-infected mouse, all neurons within a particular neuroanatomical region appear to release PrP ***(21)***. In some brain areas, diffuse staining patterns of PrP accumulation, seen prior to terminal

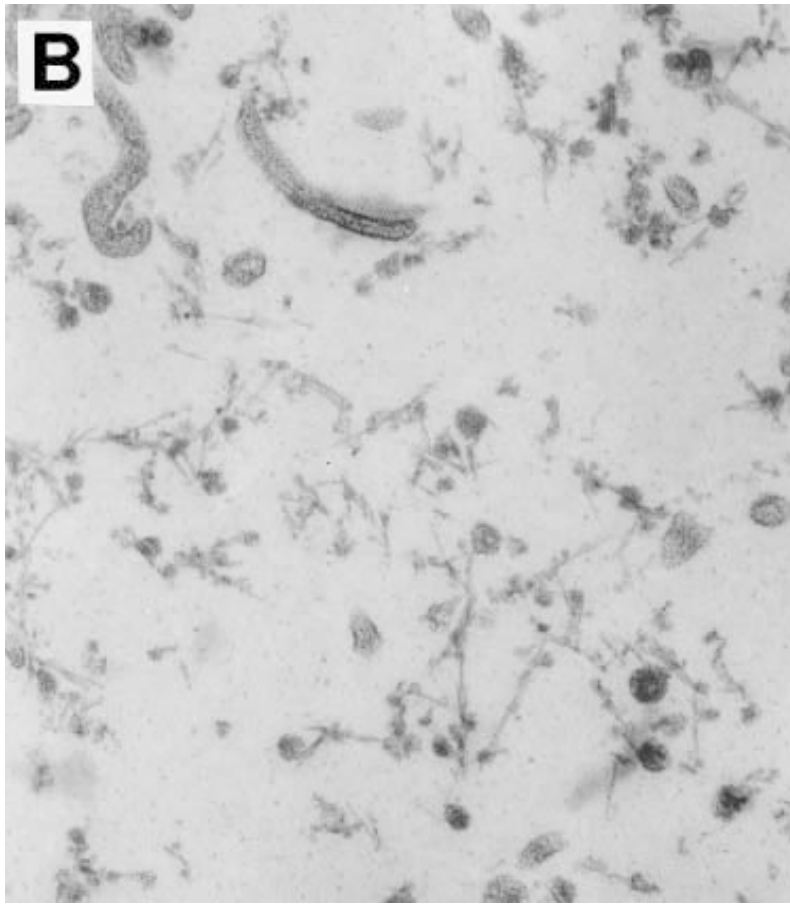

Fig. 5 *(continued from facing page)* **(B)** The ventricular deposit shown in (**A**), but at higher magnification. This confirms the fibrillar nature of this ventricular deposit. Stained with uranyl acetate and lead citrate.

disease, may become dense, localized, granular patterns of PrP accumulation at terminal disease. Granular patterns of PrP accumulation correlate ultrastructurally with widespread PrP aggregation and fibrillization within the extracellular space. This is invariably associated with marked gliosis, and sometimes with widespread neuronal loss. PrP release from ependymal cells in sheep, and in 263K infected hamster, forms amyloid fibrils within the extracellular space. Ependymal cells release PrP from their ventricular border, and subsequently form fibrils within the ventricular space (**Fig. 5**), which suggests that the requirement for extracellular matrix components to form fibrils may not be obligate.

Intracellular PrP is found within macrophages of lymph nodes and in astrocytes, microglia and in Kohler cells of the choroid plexus and ventricle *(15,43)*. All of these cells are known to have phagocytic properties, and to be involved in managing removal of degenerate or toxic products from tissue. In all of these cell types, PrP was located to lysosomes. We suggest that abnormal or excess PrP may elicit microglial and macrophage activation. These cells are then stimulated to internalize abnormal or excess PrP from the surrounding extracellular space and to initiate degradation of the disease-specific form of PrP. Evidence is emerging, from our studies of sheep lymph nodes, that the PrP present in the lysosomal compartment may express different epitopes from PrP in extracellular sites.

Disease-specific intracellular accumulations of PrP are also found in neurons but the subcellular localization of such accumulations has not yet been achieved by electron microscopy and the significance of such intracellular accumulations is unclear. However, in the ME7 model, there is some evidence that PrP may accumulate within neuronal cytoplasm at early stages of infection, but is present extracellularly and absent from the intracellular compartment at later stages of disease. Such a change in subcellular localization could be caused by altered regulation of PrP production at early stages of infection *(44)*.

4. Relationship Between Pathological Change, PrP Accumulation, and Disease

Clinical scrapie has been described in sheep and beige mice in the absence of vacuolar changes *(8)*. Similarly, in the Chediak Higashi strain of mink, transmissible mink encephalopathy infection will cause clinical disease, but does not result in vacuolation *(45)*. For these and other reasons, vacuolation does not appear to correlate with clinical disease *(46)*. Fibrillar PrP, as described in **Subheading 3.**, will damage neuronal tissue and induce gliosis, but not all TSEs result in electron microscopically visible amyloid deposition. However, not all TSEs have amyloid plaques, and the patterns of plaque formation in most of those that do develop plaques is neither sufficiently extensive nor intense to cause neurological deficiency. For example, cerebrovascular amyloid formation in sheep is inconsistently found, and only infrequent plaques are found within individual brains. In BSE, clinical signs include loss of ruminal motility and bradycardia *(47)*. These clinical deficits are probably caused by abnormalities of the vagal nucleus, solitary tract, and nucleus ambiguus, sites which, in BSE, are the first and most severely affected nuclei for vacuolar changes and PrP accumulation *(12,13)*. Ultrastructural examination of these neuroanatomical nuclei fails to show any amyloid fibril formation or aggregated forms of PrP, and in only some cases is there gliosis (personal observa-

tions). Several studies have shown that apoptotic neuronal loss is a feature of scrapie ***(48)***, which may occur early, during the incubation period ***(49)***. To determine whether PrP accumulation is directly related to clinical disease we have counted neurons in several neuroanatomical sites of BSE-affected brains ***(50,51)***. Although neuronal loss did occur in some sites, such as the vestibular neurons, and may therefore be involved in balance deficiencies, neuronal loss is not a feature of the N. vagus or N. ambiguus, and cannot therefore be implicated in all clinical deficits ***(51)***.

In a further model of murine scrapie, we counted dendrites and synapses in the hippocampus of ME7-infected murine scrapie brains, in a model in which there is profound loss of pyramidal neurons within the CA1 sector ***(52)***. These studies revealed that loss of synapses and axon terminal degeneration occurred at approx 34–39% of the incubation period (%IP), but neuronal loss did not occur until 72%IP. The nature of the axon terminal degeneration was similar to that seen in excitatory amino acid intoxications. Preamyloid PrP within the extracellular space may perturb synaptic transmission in some sensitive neuroanatomical regions. These results suggest that neuronal loss may occur following deafferentation of neurons, as a consequence of synaptic loss subsequent to PrP accumulation within the extracellular space. Some studies of PrP^{sen} localization have suggested that PrP^{sen} may be located in the presynaptic membrane ***(53)***. These results would be consistent with the above observations, but the ultrastructural localization demonstrated showed only weak and nonspecific labeling, which therefore needs further confirmation. That significant synaptic loss precedes neuronal loss may indicate that synaptic loss is the principal cause of neurological deficits. The axon terminal degeneration described in the ME7 hippocampus is also found at other sites where there is widespread preamyloid PrP accumulation, both at other sites of the ME7-infected brain and in other murine models and in the thalamus of FSE-affected cats. However, we have been unable to find this lesion in BSE-affected brainstems. Although synaptic counting studies have not yet been widely performed in TSEs, it seems unlikely that synaptic loss can account for all the clinical deficits. Therefore, aggregated or fibrillar PrP, neuronal loss, synaptic loss, and possibly vacuolation and gliosis have some significance regarding the development of clinical disease, but that there may be yet other factors that are involved, and possibly may be of major significance in neurological dysfunction.

In the ME7 murine model described above, axon terminal loss is present (34%IP) at up to 80 d before neuronal loss (72%IP) and 140 d before terminal disease. Although PrP accumulation can be detected by immunohistochemistry in the stratum radiatum at 42%IP, at this stage, the accumulation is slight, and it is by no means clear that the amount of PrP accumulation is sufficient to

cause pathological change. PrP^{res} can be detected by Western blotting at about 80 dpi (32%IP) in whole brains of scrapie-infected mice, but disease-specific accumulations of PrP of unknown protease resistance can be detected in both hippocampus and thalamus from 60 dpi (24%IP) by immunocytochemistry ***(44)***. Although questions remain about the relative sensitivity of these techniques, these findings open the possibility that PrP is first released from cells as PrP^{sen}, which only later acquires protease resistance. A human recombinant PrP, spanning residues 91–231, has recently been shown to undergo a reversible conformational change between a protease-sensitive soluble α-helical form and a protease-resistant fibrillogenic β-pleated sheet form, depending on the ionic strength of the solution ***(54)***.

Whatever the relative sensitivities of Western blotting and immunohistochemistry, infectivity levels considerably precede increased PrP accumulation. In ME7-infected CVF1 mice, significant levels of infectivity are present from at least 20 d before the first detection for PrP by immunocytochemistry and the infectivity curves and subjectively assessed levels of PrP accumulation are different, suggesting that infectivity and PrP accumulation may not be directly linked. Similar dissociations between infectivity and PrP^{res} accumulation have been recorded by others working with different rodent models of disease ***(55,56)***.

5. PrP^{res} Processing in Cultured Cells and In Vivo Observations

PrP^{sen} is attached to the exterior of the cell plasma membrane by a glycosyl phosphadidyl inositol (GPI) anchor. Studies of scrapie-infected cell culture lines have shown that PrP^{res}, in contrast to PrP^{sen}, cannot be released from the cell surface on treatment by a bacterial phosphadidyl inositol-specific phospholipase that specifically cleaves the GPI anchor ***(57,58,59)***. The visualized location of disease-specific PrP accumulations seen in the electron microscope appears to be in conflict with the in vitro studies. If PrP^{res} remains bound to the cell surface, it is difficult to explain the apparent diffusion of disease-specific PrP away from scrapie-infected cells through the extracellular space. It is also difficult to explain why PrP^{res} is released from the surface of infected ependymal cells into the ventricular space to form fibrils, unless, that is, the initial PrP released by scrapie-infected cells is in the form of PrP^{sen}.

The occurrence and distribution of clathrin-coated pits provides a possible discrepancy between in vitro observations of brain and spleen and studies of cell culture systems. Endocytosis of a number of cell surface receptors (e.g., transferrin) takes places via clathrin-coated pits. These receptors are transmembrane structures with a cytoplasmic domain necessary for the assemblage of clathrin lattices on the cytoplasmic face of the plasma membrane. In cultured scrapie-infected cells, it has been suggested that PrP^{C} is endocytosed via

clathrin-coated pits ***(59,60)***. However, the PrP^{C} molecule does not appear to have a cytoplasmic component with which to bind clathrin. Our subcellular studies of scrapie-infected cells do not show increased clathrin-coated pit activity in association with early PrP release (?PrP^{sen}) around neurons or follicular dendritic cells. However, markedly increased clathrin-coated pit activity is seen in association with aggregated and fibrillar forms of PrP (PrP^{res}). These findings suggest that aggregated PrP^{res} binds to a transmembrane receptor, which then elicits coated-pit activity.

6. Features of Amyloidosis

Amyloid is defined as an extracellular glycoprotein complex that is deposited in tissue. Amyloid fibrils are 7.5–10 nm-diameter, rigid, nonbranching, hollow-cored tubules of indeterminate length. When examined by X-ray diffraction, these fibrils have a characteristic β-pleated sheet configuration. This macromolecular helix of 100 nm periodicity, formed from two β-pleated sheet micelles, is responsible for the resistance of amyloid fibrils to solubilization and to proteolytic digestion. Amyloid fibrils are derived from at least 15 different proteins (amyloid precursor proteins), which may be normal or abnormal variant proteins. In most amyloid disease, cell-free production of amyloid fibrils from precursor proteins has been found.

P component is a doughnut glycoprotein present in amyloid. The presence of P component in PrP amyloid is unknown. All classical amyloids contain a matrix proteoglycan. In amyloid-associated lesions of human brain, heparan sulphate co-localized with amyloid ***(61,62)***. PrP-associated deposits also contain heparan sulphates ***(42,63)***.

Several groups have previously noted that the properties and distribution of PrP^{res} are typical of classical amyloidoses and show features similar to the accumulation of bA4 Protein of Alzheimer's disease ***(7,38,64,65)***. As observed or inferred from our electron microscopy studies, the subcellular localization and onset of protease resistance of PrP is also similar to that observed or predicted for other amyloid proteins. That is, in common with other amyloid proteins, PrP is released as a preamyloid protein, probably as a protease-sensitive molecule, which aggregates, forms β-pleated sheets, binds sulphated proteoglycans, acquires partial protease resistance, and damages tissue, as aggregated isoforms or as amyloid. The N-terminal truncation of PrP does not appear to occur in vivo in the nongenetic forms of TSEs but may do so in human genetic TSEs, suggesting that there may be different amyloid proteins in different diseases. These findings therefore support the suggestion that the transition between PrP^{sen} and PrP^{res} occurs within the extracellular space after release of the normal protein by scrapie-infected cells. However, there is some evidence of strain dependent variation in processing PrP within individual cells

and differences in glycoform patterns of PrP^{res}, are found following infections with different strains of agent infection. The different glycoform patterns may be because of infection of different subpopulations of neurons, which express different proportions of the three glycoforms of PrP, or by altered metabolism of PrP in scrapie-infected cells.

If the different glycoforms of PrP^{res} are secondary to infection-induced abnormal accumulation or polymerization of a normal protein, then the nature of the infections of the TSEs remains unexplained. There is also some evidence to suggest that there are some disease models in which PrP accumulation is either not present, or is present at low levels. Many previous studies have searched for and failed to find conventional micro-organisms in tissues of TSE-affected animals and human beings. And, indeed, most previous reports of viral-like organisms have now been shown to be artifacts or other pathological changes associated with TSE infection. There remains, however, one unexplained structure, which may be of significance.

7. Tubulovesicular Particles

So-called tubulovesicular particles or TVBs, perhaps better named "scrapie-associated particles," because they are not always visualized in tubular form, were first described in the brains of scrapie-infected mice by David-Ferreira et al. ***(66)***. TVBs have ever since been regularly, if inconsistently, described in electron microscopic studies of the brains of various experimental and naturally TSEs ***(67)***. TVBs have been described in most rodent models of scrapie, in natural sheep scrapie, CJD, GSS, and in BSE ***(67)***. We have also seen such particles in feline spongiform encephalopathy (**Fig. 6**), but these particles have not been reported in spleens or in infected brain cell cultures.

The molecular structure of TVB is unknown, but staining of thin sections by ruthenium red enhances their appearance, suggesting that they contain glycosyl residues ***(68)***. Murine brain tissue, infected with ME7, 87V, or 22CH scrapie isolates, and stained by immunogold methods, were negative for PrP epitopes, using two different anti-PrP sera ***(17,69)***. Descriptions of the size and shape of the particles differ, probably because of differences in postmortem delay and different fixation methods. Most reports describe the particles as spherical, with a diameter of between 30 and 35 nm (which is significantly more than the smallest circoviruses, at around 17 nm). The particles are pleomorphic, with some appearing as short rods or ellipsoids. They can be found in axon terminals, but are seen mostly in dendrites.

We have determined the number and density of TVBs present at selected stages of the incubation period in the F1 cross of C57 and VM mice infected with ME7 scrapie, and related their occurrence to the temporal onset of various of pathological changes. ***(52)***. As described above, in this model, the earliest

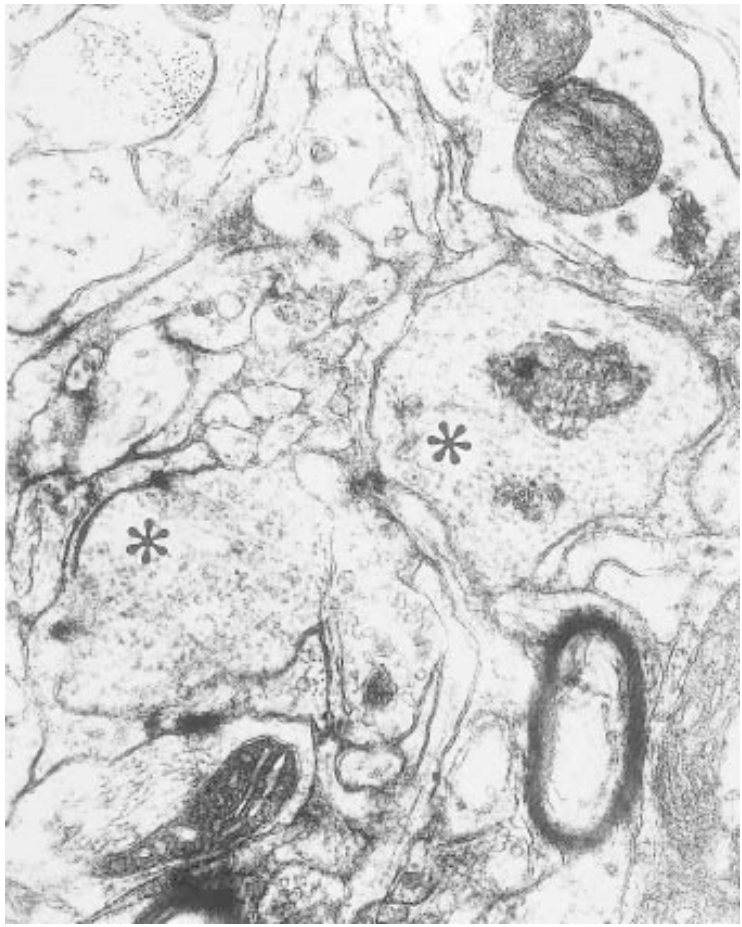

Fig. 6. The thalamus from a naturally occurring case of feline spongiform encephalopathy is shown. Two dendrites (asterisks) contain large numbers of spherical or elliptical structures: so called tubulovesicular bodies. The size of these bodies can be compared to synaptic vesicles in an adjacent axon terminal (star) or cross-sectioned microtubules (arrowheads). Stained with uranyl acetate and lead citrate.

changes seen, at about 100 dpi, are a degeneration of axon terminals and synaptic loss. Terminal disease is around 250 dpi. In blind coded trials, the number of tubulovesicular particles were counted and their density was estimated in serial pairs of electron micrographs taken from the stratum radiatum at 84, 100, 126, 154, and 181 dpi, and from four normal brain-inoculated control mice. Tubulovesicular particles were present from 98 dpi, and the density of particles increased with increasing incubation period. The early occurrence of tubulovesicular particles, before the presence of significant pathology, argues

that tubulovesicular particles are a part of the primary disease, and are not epiphenomena ***(70)***.

The data currently available, therefore, indicate that TVBs are specific to the TSE, and, although they are not composed of PrP, have been found in all forms and animal models of TSE so far examined. They are present at or before the earliest stages of pathology, increase in correlation with infectious titer, and share some of the structural features of viruses. However, they have not yet been found in scrapie-infected cell culture lines or in spleens, which is an absolute requirement, if they are to be considered as a possible infectious cause of the TSEs.

8. Conclusions

Our studies of several experimental murine disease models of scrapie and naturally occurring animal diseases show that, despite variation in the light microscopic appearance of the forms of PrP accumulation and the neuroanatomical location of cell type at which it occurs, there is a consistent sequence of events, consisting of plasmalemmal PrP release and extracellular PrP accumulation, followed by aggregation and fibrillization. Some of the pathology of the TSEs, such as gliosis and neuronal apoptosis, is associated with these aggregated forms of PrP, but this is insufficient to explain all the pathology of the TSE or clinical disease. In particular, clinical signs relating to specific neuroanatomical areas of the BSE and sheep scrapie brain can be identified, in which there is neither fibrillar PrP nor any subcellular features of pathology so far identified. The TEM features associated with abnormal PrP accumulation are closely similar to, or indistinguishable from, amyloid biology. Neither abnormal PrP accumulation nor vacuolation are sufficient to explain all aspects of the pathology of TSE, and factors other than abnormal PrP accumulation may be necessary to explain infectivity. TVB have been found ultrastructurally in many TSEs; they are not directly associated with PrP accumulation or amyloid pathology. The cause and functions of TVB are unknown, but a central role in pathogenesis cannot be discounted.

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13

Conformation as Therapeutic Target in the Prionoses and Other Neurodegenerative Conditions

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1. Introduction

Neurodegenerative conditions are increasing in prevalence as the average human life expectancy rises. Alzheimer's disease (AD) is the fourth commonest cause of death in the United States; the recent outbreak of new variant Creutzfeldt-Jakob disease (nvCJD) has raised the specter of a large population being at risk to develop this prionosis. The pathogenesis of many neurodegenerative diseases is now recognized to be associated with abnormalities of protein conformation. A common theme in these disorders is the conversion of a soluble normal precursor protein into an insoluble, aggregated, β-sheet rich form that is toxic. In AD, a critical event is the conversion of the normal, soluble Aβ (sAβ) peptide into fibrillar Aβ, within neuritic plaques and congophilic angiopathy ***(1)***. Similarly, in the prionoses, the central event is the conversion of the normal prion protein, PrP^{C}, to PrP^{Sc} ***(2)***. An increased β-sheet content characterizes both Aβ and PrP^{Sc}.

AD and many of the prionoses are forms of cerebral amyloidosis. Several other diseases also fall into this category, and include familial British dementia ***(3)***, familial Danish dementia, and familial Hungarian amyloidosis ***(4)***. In these cerebral amyloidoses, either a mutation occurs in a systemically expressed precursor protein, which increases the propensity of the entire protein and/or a degradation fragment to adopt a β-sheet conformation, or this conformational change can occur spontaneously.

Several other neurodegenerative diseases, which are not amyloid diseases, also have a similar pathogenesis (**Table 1**). For example, Huntington's disease and the spinocerebellar ataxias are associated with increased CAG repeats in

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Table 1
Conformational Neurodegenerative Conditions Characterized by Abnormal Protein Structure

Disease	Protein	Normal structure	Abnormal structure	Location of abnormal protein accumulation
Prionoses	Prion	α-Helical and random coil	β-Pleated	Variable extracellular amyloid
Alzheimer's disease	Amyloid β	α-Helical and random coil	β-Pleated	Extracellular amyloid
Familial British dementia	Bri	Mainly α-helical and random coil	β-Pleated	Extracellular amyloid
Familial Hungarian dementia	Trans-thyretin	Mixture of α-helical and β-pleated	β-Pleated	Extracellular amyloid
Frontotemporal dementia	tau	Soluble	Aggregated	Cytoplasmic
Huntington's	Huntingtin	<35 CAG repeats	>36 CAG repeats	Nuclear
Parkinson's	α-Synuclein	Soluble	Aggregated	Cytoplasmic
Spinocerebellar ataxia	Ataxin	Few CAG repeats	Many CAG repeats	Nuclear
DRPLA	Atrophin-1	<36 CAG repeats	>49 CAG repeats	Nuclear
SBMA	Androgen receptor	<36 CAG repeats	>40 CAG repeats	Nuclear

DRPLA, dentatorubral and pallidoluysian atrophy; SBMA, spinal and bulbar muscular atrophy.

the Huntington and ataxin genes respectively ***(5,6)***. These CAG repeats result in increased lengths of glutamine residues, which enhance the propensity for the mutant protein to aggregate and form intracellular neuronal inclusions that produce toxicity. The recognition of the importance of abnormal conformation in these disorders has led to the development of both anti-β-sheet compounds and immunological approaches that affect the conversion into the pathological conformer and/or the clearance of the disease-associated proteins. These approaches hold promise as potential therapeutic strategies in this category of illness.

2. β-Sheet Breaker Peptides and the Prion Protein

A number of compounds have been tried in the treatment of prion diseases, including Congo red ***(7,8)***, anthracyclines ***(9)***, amphotericin B ***(10,11)***, and sulphated polyanions ***(12)***. Some of these have been shown to delay the incubation times of animals infected with scrapie, but these agents have limitations in terms of toxic effects and/or unfavorable pharmacokinetic properties. We have recently designed a number of compounds that interact with the PrP^{Sc} structure and act as β-sheet breakers ***(13)***. These are short, synthetic peptides, which, because of sequence homology, bind to PrP. Prolines (Pros) were introduced into these short peptides, since prior data suggests that the presence of these residues inhibits a β-sheet conformation ***(14–16)***. Pros are incompatible with a β-sheet conformation for a number of reasons, including the peptidyl–prolyl bond induced by the Pro ring does not fit with the peptide bond geometry found within β-sheet motifs, and the Pro ring sterically hinders the β-sheet bonding network. The PrP sequence picked for designing the β-sheet breaker corresponded to residues 115–122, because this region has been implicated in the conversion process of PrP^{C} to PrP^{Sc} ***(17–19)***.

A number of different PrP homologous peptides (some of which are shown in **Table 2**) were first screened for inhibitory activity on the conversion of PrP^{C} to PrP^{Sc} using an in vitro system. Synthetic peptides, corresponding to PrP residues 109–141, can reproduce some of the properties of PrP^{Sc} in vitro ***(17,18,20)***. The authors determined the ability of these various candidate β-sheet breaker peptides to inhibit amyloid-like fibril formation of PrP109–141, using a fluorometric assay based on the fluorescence emission of thioflavine T ***(21,22)***. Using this assay, a 13-residue peptide (iPrP13) had the greatest β-sheet-breaking capability (**Fig. 1**).

Using this peptide we were able to show that the proteinase K sensitivity of extracted mouse PrP^{Sc}, human PrP^{Sc} extracted from sporadic CJD patients, or from a nvCJD patient, was increased, in a concentration-dependent fashion, by iPrP13 ***(13)***. The in vivo effect of iPrP13 was also tested using the mouse-adapted scrapie strain 139A. Incubation time assays were done using three different 10-fold dilutions of extracted 139A PrP^{Sc}, in the presence or absence of an equimolar concentration of iPrP13. At each dilution, one group of mice was injected with untreated and nonincubated PrP^{Sc}, a second group was inoculated with PrP^{Sc} that was incubated for 48 h alone, a third group was inoculated with PrP^{Sc} and iPrP13 without incubation, and a fourth group was inoculated with PrP^{Sc} and iPrP13 following 48 h incubation. The iPrP13 induced a substantial delay in the appearance of disease (**Table 3**).

The above results suggest that β-sheet breakers may have therapeutic potential in the prionoses. Such an approach (**Fig. 2**) may have applicability to any

Table 2
The Sequence of β-Sheet-Breaker Peptides and Control Peptides Used

Peptide	Sequence
iPrP13	DAPAAPAGPAVPV
iPrP11	DAAAPAGAPVV
iPrP10	DAPAAPAVPV
iPrP9	DAAPAAPVV
iPrP8	DAPAAPVV
iPrP7	DAAAPVV
iPrP5	AAPVV
CP1	GYITVAAVFRG
CP2	PAADVPPAAV
PrP101-119	KPSKPKTNMKHMAGAAAAG
PrP109-122	MKHMAGAAAAGAVV

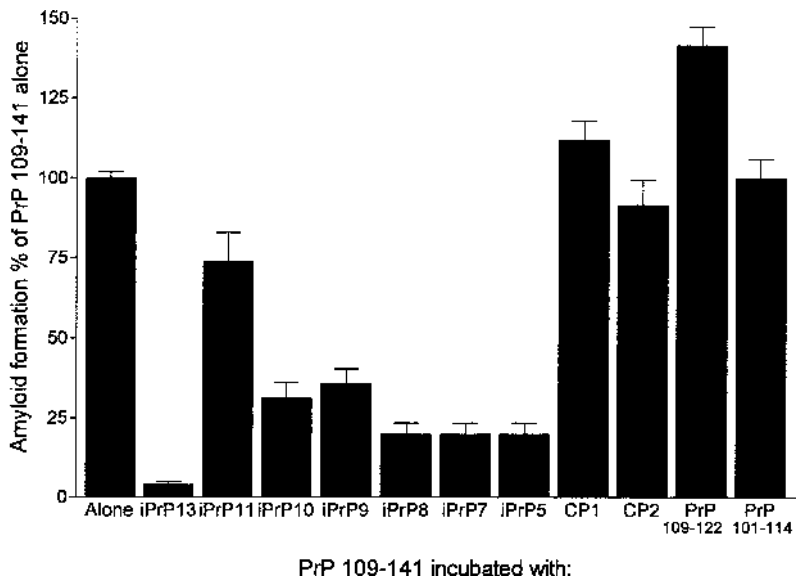

Fig. 1. A number of different β-sheet-breaker candidate peptides (*see* **Table 2**) were tested for inhibition of amyloid-like fibril formation by synthetic PrP109–141. 30 μg aliquots of PrP109-141 were incubated alone or with a 10 fold molar excess of the various peptides in 30 μL 0.1 *M* Tris-HCl, pH 7.4, for 7 d at 37°C. Fibrillogenesis was quantitated by thioflavine-T fluorometic assay.

Table 3
In Vivo Effect of iPrP13 on the Incubation Times of Mouse-Adapted Scrapie Strain 139A

	Incubation time (days)			
PrP^{Sc} dilution	PrP^{Sc} alone $T = 0$	PrP^{Sc} alone $T = 2$ d	PrP^{Sc} + iPrP13 $T = 0$	PrP^{Sc} + iPrP13 $T = 2$ d
100	129 ± 0	136 ± 5	143 ± 3 *	148 ± 2*
1000	145 ± 6	141 ± 7	159 ± 2 **	176 ± 9*
10000	173 ± 12	162 ± 9	185 ± 13	225 ± 26**

Incubation time assays were done using three different 10-fold dilutions of extracted 139A PrP^{Sc}, in the presence or absence of an equimolar concentration of iPrP13. At each dilution, one group of mice was injected with untreated and nonincubated PrP^{Sc}, a second group was inoculated with PrP^{Sc} that was incubated for 48 h alone, a third group was inoculated with PrP^{Sc} and iPrP13 without incubation, and a fourth group was inoculated with PrP^{Sc} and iPrP13 following 48 h incubation. The iPrP13 induced a substantial delay in the appearance of disease (*indicates $P < 0.05$ and ** indicates $P < 0.06$ vs PrP^{Sc} at the same dilution and incubation time).

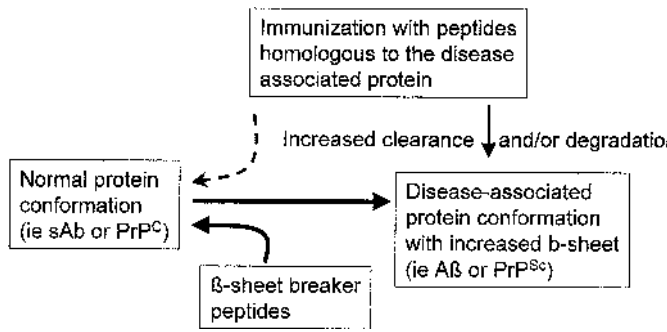

Fig. 2. Hypothetical scheme for anticonformational therapeutic agents. β-sheet breakers can function both to inhibit the formation of a β-sheet conformation, and to return the abnormal conformation to its physiological form ***(24,13)***. Immunization with the peptides homologous to the disease-associated protein can act to increase its clearance and/or degradation. Alternatively, antibodies to the disease-associated protein may also act to inhibit the conformation change to increased β-sheet, as has been shown in vitro for some anti-Aβ antibodies, which disaggregate Aβ peptides ***(37)***.

of the conformational diseases listed in **Table 1**. However, significant disadvantages of iPrP13 are that, as a short peptide, it is likely to be subject to extensive proteolytic degradation, and that it has poor permeability of the blood–brain barrier. These difficulties could be overcome by designing peptidomimetic or pseudopeptides, based on the structure of iPrP13. This β-sheet-

breaker concept also does not need to be limited to agents that are homologous to the physiological precursor protein. In that light, a recent report, using porphyrin and phthalocyanine compounds, showed an increase in the survival times following inoculation of PrP^{Sc} in experimental animals ***(23)***. The activity of these compounds was, like iPrP13, correlated with in vitro inhibition of PrP^{Sc} formation.

3. β-Sheet-Breaker Peptides and Amyloid β

Major features of AD are the deposition of Aβ in the form of neuritic plaques and congophilic angiopathy, in which it is fibrillar and has a high β-sheet content. The Aβ peptide also exists as a normal peptide in biological fluids, where it is called "soluble Aβ" (sAβ), and is thought to have a more random coil and/or α-helical secondary structure. Hence, a critical event in the pathogenesis of AD is the conformational change of sAβ to Aβ ***(1)***. This conformational change is analogous to the PrP^{C}-to-PrP^{Sc} transition. Similar in concept to the authors' design of iPrP13, the authors have also designed Aβ homologous peptides, with Pro residues that inhibit a β-sheet conformation. Using an in vitro assay of Aβ fibrillogensis, a five-residue peptide, iAβ5, was found to have the greatest activity ***(15)***. In a rat brain model of Aβ amyloidosis, iAβ5 inhibited fibril formation when administered at the same time as an intracerebral inoculation of Aβ1–42. In rats in which the Aβ1–42 was injected without iAβ5, large Congo red-positive, fibrillar deposits formed ***(16)***. These results indicated that peptides such as iAβ5 could be used to prevent Aβ deposits from forming. More recently, we have also used the rat model of AD to show that iAβ5 can reverse existing deposits of Aβ1–42 (**Fig. 3**; ***24***). In this experiment, Aβ1–42 was first stereotactically injected into the amygdala of the rat and allowed to form fibrillar deposits over a period of 8 d. At that point, the rats were injected with iAβ5, control peptides, or vehicle, followed by histological examination 8 d later. The rats injected with the iAβ5 showed evidence of dissembly of the Aβ fibrils that had formed in vivo ***(24)***. A number of other compounds have also been shown to dissemble Aβ fibrils, using in vitro assays, such as melatonin ***(25,26)***, nicotine ***(27)***, apolipoprotein J ***(28)***, anthracycline ***(29)***, rifampicin ***(30,31)***, hexadecyl-*N*-methylpiperidinium ***(32)***, and Congo red ***(33)***. It remains to be determined if these will be active in vivo. With the current availability of various transgenic mouse models of AD that develop cerebral amyloid deposits, many of these compounds are now being tested for both prevention of amyloid formation, and for the disassembly of existing lesions.

4. Immune Response in the Prionoses and Alzheimer's Disease

It has recently been shown that immunization of transgenic mice with AD-related neuropathology, using fibrillar Aβ1–42 as an antigen, reduces or

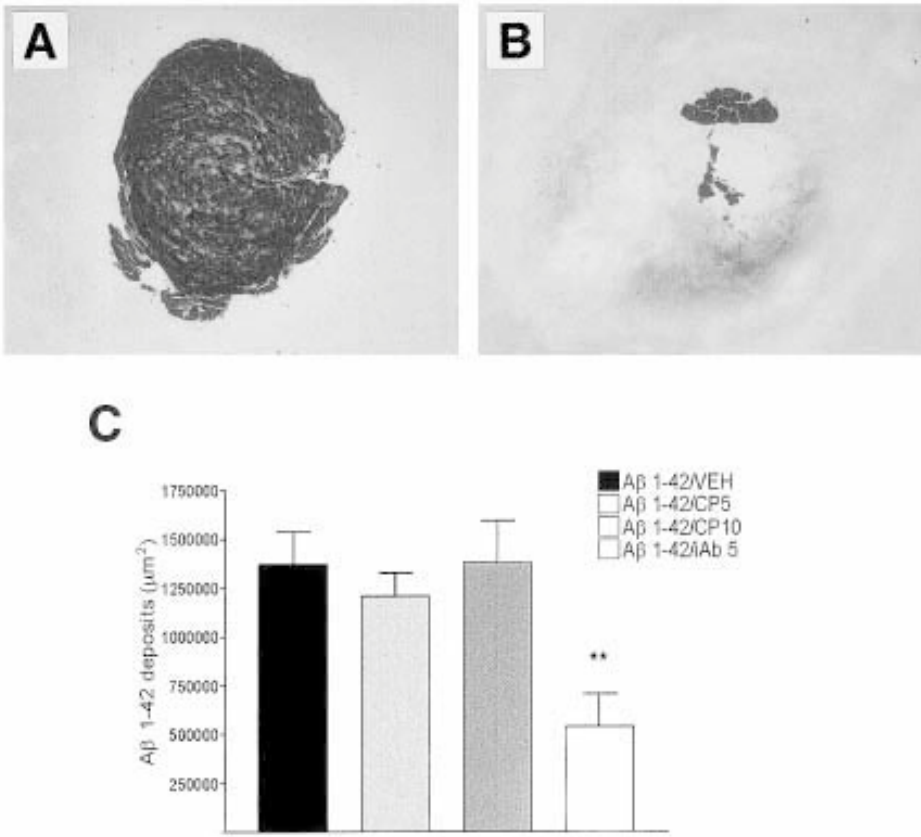

Fig. 3. Specific disassembly of Aβ fibrils in vivo by iAβ5, and subsequent reduction of cerebral Aβ deposition. Aβ-stained coronal sections (×100, original magnification) through the amygdala at the injection site, in rats treated with **(A)** Aβ1–42/vehicle (VEH) and **(B)** Aβ1–42/iAβ5. Intra-amygdaloid injection of VEH or iAβ5 was performed 8 d following injection of Aβ1–42 into the same brain region, and the rats were killed 8 d later. **(C)** Quantitative analysis of Aβ deposits at 16 d postoperatively in rats injected first with Aβ1–42 (5.0 nmol), followed 8 d later with an injection of iAβ5 (LPFFD), control peptides (CP5 [ETRGD], CP10 [ISEVKMDAEF]) (200.0 nmol), or VEH. Deposit area was measured by image analysis of Aβ immunoreactive sections. Each bar represents the mean + SEM of $N = 8–9$ rats. Effect of iAβ5 relative to the other treatment groups: ** $P < 0.01$.

prevents cerebral Aβ amyloid deposits ***(34)***. The transgenic mice used in this experiment overexpress mutant human APP, and progressively develop Aβ cerebral amyloid deposits in an age- and brain-region-specific manner ***(35)***.

When animals were inoculated with fibrillar Aβ1–42 at 6 wk, prior to the development of AD-related pathology, cerebral amyloid deposition was essentially prevented, but animals inoculated at 11 mo, with substantial amyloid deposits, had the AD-related pathology greatly reduced. These inoculations resulted in the generation of high anti-Aβ antibody titers. The protective effect on cerebral amyloid deposition could be related to an inhibition of the conformational change from sAβ to Aβ (similar to the β-sheet-breaker peptides discussed above), or other effects, such as increased clearance of Aβ and/or blocking of Aβ/immune cell interactions, which are involved in amyloid formation. Prior in vitro data suggests that at least some anti-Aβ antibodies can inhibit fibrillar aggregation of these peptides ***(36,37)***.

These experiments suggest that modulation of the immune system against aggregated, disease-associated proteins may represent a novel therapeutic approach. Furthermore, in another model system, it has recently been shown that an oral vaccine is effective at prevention of experimental stroke and epilepsy ***(38)***, illustrating the feasibility of a vaccination strategy that targets brain proteins. The importance of the immune system in the pathogenesis of AD has been further underscored by the recent reports of a genetic linkage between common population polymorphisms of the interleukin-1α and interleukin-1β genes ***(39,40)***. Interleukin-1 is an acute-phase proinflammatory cytokine, which has been implicated in neuroinflammatory pathways resulting from Aβ cerebral deposition. Many laboratories, including that of the authors, are currently testing a number of different Aβ epitopes in various transgenic AD mouse models, to assess this as a therapeutic approach. Our preliminary results using a variety of Aβ peptide epitopes for immunization confirm that such a 'vaccination' approach results in a dramatic reduction in amyloid burden in transgenic AD mice. However, it remains unclear whether these interventions will be beneficial or detrimental to natural disease progression. Extensive evidence has implicated inflammatory pathways in AD ***(41)***; however, this has been previously proposed to be associated with neuronal damage and death related to complement activation, free-radical damage and microglial activation ***(41–44)***. Indeed, a number of studies have shown that patients who have a long history of taking anti-inflammatory agents have a lower incidence of AD ***(45–47)***. Hence, whether experimentally induced inflammation will be beneficial or harmful for AD is likely to depend on the type and extent of response generated by the various immunization protocols used.

Modulation of the immune system may also have the potential to be beneficial for the prionoses. The immune system has long been recognized to have an unusual role in prion infections. There are no specific humoral or immune responses against prions following infection ***(48,49)***; in fact, the immune system appears to help, rather than impair, the propagation of prions following

peripheral exposure. Although PrP^C is expressed at various levels in most tissues of the body, infectivity multiplies only within the nervous system and in the peripheral lymphoid organs ***(50)***. Mice with severe combined immunodeficiency (SCID) illustrate the paradoxical role of the immune system in prion infections. These mice are partially resistant to scrapie, after intraperitoneal or subcutaneous inoculation, in contrast to immunocompetent mice of the same strain, and to immunologically reconstituted SCID mice ***(51,52)***.

The nature and actions of the immune cell type(s) that support prion infection still remains unclear. The essential controversy concerns the respective roles of lymphocytes and follicular dendritic cells (FDCs) in the replication of prions and their transport to the nervous system. After fractionation of spleen cells by density gradient centrifugation, the highest infectivity, between the second and ninth weeks following mice inoculation with scrapie agent, was found in lymphocytes ***(53)***. However, it may be that this cell fraction also contained FDC, because of their close interactions with B-lymphocytes.

Ionizing radiations did not influence mouse susceptibility to scrapie, suggesting that quiescent cells may principally support prion replication ***(54)***. Because the scrapie susceptibility of SCID mice, after reconstitution with hematopoietic precursors, appears to be dependent on the restoration of a normal lymphoid architecture ***(51)***, and PrP^{Sc} is detected in FDC of CJD infected mice ***(55)***, FDCs are likely to be involved in the replication and accumulation of the scrapie agent. However, the occurrence of scrapie agent within FDC could reflect their potent antigen-capturing function ***(55)***. Tumor necrosis factor receptor-1-knockout mice, which lack FDC and germinal center reaction, have a normal susceptibility to scrapie after peripheral inoculation; mature B-lymphocytes, rather than other hematopoietic cell lineages, are required for the neuroinvasion ***(56)***. However, such results were not confirmed with another model of FDC deficiency: the tumor necrosis factor α-knockout mouse ***(57)***. A key role of FDC in PrP^{Sc} replication is strongly supported by recent results showing that the expression of PrP^C by FDC, but not by hematopoietic lineages, is required for scrapie susceptibility after peripheral inoculation ***(57)***.

Regardless of the question of which cells of the immune system are involved in prion propagation, it is clear that immunomodulators can influence susceptibility to scrapie. Mitogenic stimulation by phytohemagglutinin or bacterial lipopolysaccharide made mice susceptible to doses of prions that are otherwise ineffective, and reduced the incubation time after peripheral inoculation by nearly 20% ***(58)***. On the other hand, administration of high doses of prednisone, immediately before and after intraperitoneal inoculation of mice with a scrapie-infected brain homogenate, resulted in prolonged incubation periods ***(59)***. Given the encouraging results in AD model transgenic mice ***(34)***, as discussed

above, the question is raised whether a similar approach may also work in prion infection, using PrP^{Sc} peptide analogs as immunogens. Such experiments are currently ongoing.

5. Conformation as a Therapeutic Target

There is growing evidence that numerous neurodegenerative conditions have the same underlying pathogenetic mechanism, namely, a change in protein conformation in which the β-sheet content is increased. In AD, amyloid deposition, in the form of neuritic plaques and congophilic angiopathy, is driven by the conversion of sAβ to Aβ; in the prionoses, the critical event is the conversion of PrP^{C} to PrP^{Sc}. In AD and in the prionoses, as well as in other cerebral amyloidoses (such as familial British dementia ***[3]***), the abnormal β-sheet protein accumulates in the extracellular space. We suggest that this common conformational theme in these disorders, and the extracellular localization of the accumulating abnormal protein, make them highly amenable to therapeutic approaches based on experimental manipulation of protein conformation and clearance. We have outlined the design of a number of β-sheet breakers for both Aβ- and PrP^{Sc}-related deposits. These initially designed peptides can serve as a starting point for further peptidomimetic or pseudopeptide derivatives, which can have optimized biological and pharmacokinetic properties. In addition, immune system activation, directed against the abnormal β-sheet peptide, can serve as a β-sheet breaker and/or to increase the clearance of the disease-associated protein. These conformationally based approaches appear to have the best promise for rationale therapies for this devastating group of disorders.

6. Summary

Abnormal protein conformation is increasingly being recognized as part of the pathogenesis of numerous neurodegenerative conditions. The common theme in all these diseases is the conversion of a normal cellular and/or circulating protein into an insoluble, aggregated, β-sheet-rich form that is deposited in the brain. The aggregated proteins can accumulate extracellularly, often in the form of amyloid, or intracellularly, producing inclusion bodies. These deposits are toxic, and produce neuronal dysfunction and death. A unique category of the conformational conditions are prion-related diseases (or prionoses), in which the etiology is thought to be related to conversion of the normal prion protein, PrP^{C}, into an infectious and pathogenic form, PrP^{Sc}. However, the most common of these disorders is AD, in which the central event is thought to be the conversion of normal soluble amyloid β (sAβ) into fibrillar Aβ, in the form of neuritic plaques and congophilic angiopathy. Growing understanding of the mechanisms involved in this category of disease raises the possibility of therapeutic approaches based directly on the prevention and

reversal of pathologic protein conformations. Possible approaches include synthetic β-sheet-breaker peptides, which the authors' preliminary data suggest may be useful for both AD and the prionoses, as well as for immunological approaches in which an antibody and/or cell-mediated response is triggered against the aggregating abnormal protein.

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14

Prions of Yeast

From Cytoplasmic Genes to Heritable Amyloidosis

Reed B. Wickner, Herman K. Edskes, Kimberly L. Taylor, Marie-Lise Maddelein, Hiromitsu Moriyama, and B. Tibor Roberts

1. Introduction

It was believed that only proteins could carry out enzymatic reactions, and only nucleic acids could mediate inheritance. In recent years, the work of Cech and Altman and others has shown that nucleic acids can catalyze reactions. Now it has been shown that, in yeast, proteins can mediate inheritance.

The infectious protein (prion) concept arose from studies of the transmissible spongiform encephalopathies (TSEs) of mammals ***(1)***, and several lines of evidence suggest that TSEs are indeed caused by infectious forms of the PrP protein, but the absence of definitive proof has left substantial doubt and disagreement on this point ***(2–6)***. The ease of genetic manipulation of yeast offers experimental possibilities not yet available even in the mouse system. This enabled the discovery of yeast prions ***(7)***, and has facilitated the rapid characterization of these systems. The parallels between the yeast and mammalian systems are striking. Moreover, because both of the yeast prion systems appear to involve self-propagating amyloid forms of the respective proteins, these systems may also serve as models for the broader class of diseases for which amyloid accumulation is a central feature. The discovery of the [HET-s] prion of the filamentous fungus *Podospora*, another genetically manipulable system, adds a new dimension to prion studies ***(8)***.

Genetics is not only another useful tool in these studies, but is the essential component for the study of prions. Genetic criteria for prions of yeast, and specific genetic experiments designed to rule out alternative hypotheses, have

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made a convincing case that the yeast elements [URE3] and [PSI] are prions. Biochemical studies have now begun to bring evidence for the mechanisms that underlie these prions.

Many reviews of yeast and fungal prions have appeared recently, and the reader may consult these for different emphasis in coverage and different views of the subject *(9–13)*.

2. Genetic Criteria for Prions

We use the word 'prion' to mean a protein that is infectious, by whatever mechanism. Infectious proteins of yeasts and filamentous fungi should therefore be infectious in a similar way to viruses of these organisms. Perhaps because in nature yeast mate frequently, yeast viruses are infectious only via this mating process, and yet they are widely distributed in natural isolates. When an infected cell mates with an uninfected cell, all of the meiotic and mitotic progeny are infected. Thus, the virus appears as a nonchromosomal gene (a non-Mendelian genetic element). Yeast viruses do not pass out of one cell and enter another. The thick wall of yeast cells may contribute to this fact, but the even thicker plant cell walls do not prevent extracellular spread of plant viruses. Viruses of filamentous fungi likewise only spread via cell-to-cell fusion, and are strictly intracellular entities. Infectious proteins (prions) would thus be expected to likewise appear as nonchromosomal genetic elements *(7)*.

Among nonchromosomal genetic elements, three genetic properties were proposed that should distinguish those resulting from nucleic acid replicons, such as viruses or plasmids, from prions (**Fig. 1**; *see* **ref. 7**).

2.1. Reversible Curability

If a prion can be cured from a strain, it should be possible to find a rare subclone of the cured strain in which the prion has again arisen spontaneously. The change that makes a protein infectious should occur with some low frequency in any sufficiently large population of the normal form of the molecule. In contrast, curing a plasmid (such as the yeast 2-μ DNA plasmid) or a virus (such as the killer virus) is an irreversible event. Once the virus or plasmid has been cured, it will not arise spontaneously, but can only reappear by being reintroduced from another cell (**Fig. 1**, top).

2.2. Overproduction of Protein Increases the Frequency with Which the Prion Arises

Whatever the mechanism by which prions arise, it is expected that increasing the amount of the normal form should increase the number of molecules that have the potential to undergo the prion change, and thus the frequency with which the prion formation event occurs (**Fig. 1**, middle). In contrast, there

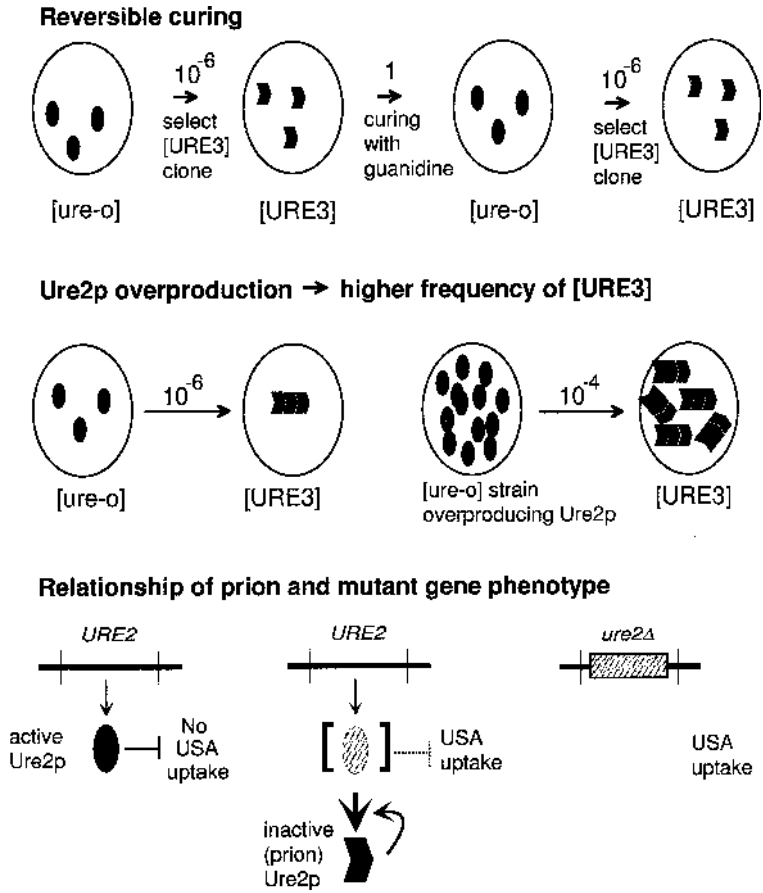

Fig. 1. Genetic criteria for a prion. To distinguish cytoplasmic genetic elements due to a nucleic acid replicon (and a chromosomal gene needed for its replication) from a prion (and the chromosomal gene encoding the protein), three genetic criteria were proposed *(7)*. (**Top**): Curing [URE3] with guanidine produces cells from which [URE3] can again arise at frequencies similar to that at which it arose from the wild-type ancestor. (**Middle**) Overproducing Ure2p increases the frequency with which [URE3] arises. (**Bottom**) The phenotype of *ure2* mutants is the same as that of [URE3] strains because both phenotypes arise from deficiency of normal Ure2p; and *URE2* is necessary for propagation of [URE3].

is no chromosome-encoded protein whose overproduction will induce a nucleic acid replicon to arise *de novo*.

2.3. Phenotype Relationship of the Prion and a Prion Maintenance Gene

If the prion causes cellular pathology because the normal form is converted to an abnormal nonfunctional form, then the deficiency of the normal form should

produce the same phenotype as that produced by mutation of the chromosomal gene for the normal form (**Fig. 1**, bottom). Furthermore, this chromosomal gene for the normal form should be necessary for the propagation of the prion. Thus, a prion would have, as one of the chromosomal genes needed for its propagation, one whose mutant phenotype was the same as that produced by the presence of the prion. This is not the relationship between a nucleic acid replicon and the chromosomal genes needed for its propagation (**Table 1**). For example, the mitochondrial DNA encodes proteins necessary for utilization of glycerol, a nonfermentable carbon source. Cells carrying mitDNA can grow on glycerol, but mutants in the chromosomal gene for the DNA polymerase responsible for replicating mitDNA lose mitDNA, and cannot grow on glycerol, which is the opposite phenotype.

3. TSEs Do Not Yet Fulfill Genetic Criteria for a Prion

None of the TSEs can yet be cured, so reversible curing is not even testable. Transgenic mice overproducing PrP have been constructed, and these mice do become sick and die as a result, but their tissues have not been shown to contain infectious material. That is, the overproduction of PrP is not, in these mice, giving rise to an infectious entity ***(14)***. PrP is necessary for the propagation of the scrapie agent ***(15)***, but the phenotype of deletion of the *PRNP* gene ***(16)*** is not at all like that of scrapie. This result is consistent with scrapie being caused by a virus or other nucleic acid replicon, but it does not rule out scrapie being a prion. The prion form of PrP presumably has a positively harmful effect on cells. Thus, the yeast prions were established, based on evidence of a type not yet available for the mammalian TSEs.

4. [URE3] Is a Nonchromosomal Genetic Element Affecting Regulation of Nitrogen Catabolism

When presented with a rich nitrogen source, such as ammonia or glutamine, yeast represses the transcription of genes whose products are involved in the utilization of poor nitrogen sources, such as allantoate, a degradation product of purine metabolism (**Fig. 2**; ***17,18)***. The chance resemblance of allantoate to ureidosuccinate, an intermediate in uracil biosynthesis, results in uptake of ureidosuccinate being controlled by the nitrogen regulation system ***(19,20)***.

Lacroute et al. isolated mutants able to take up ureidosuccinate on media containing ammonia ***(21–25)***. These mutants defined two chromosomal genes, *ure1* and *ure2*, and a nonchromosomal genetic element, [URE3]. Although the *ure1* and *ure2* mutants were recessive or partially recessive, [URE3] was dominant, and could be transferred from cell to cell by cytoplasmic mixing ***(25)***. The molecular basis of [URE3] was unclear, and studies of [URE3] lapsed for many years. However, the role of Ure2p in nitrogen regulation was found to be

Table 1
Relation of Phenotypes of Nucleic Acid Replicon, Prion and Chromosomal Genes Needed for Their Propagation

	Phenotypes			
Non-Mendelian element	Presence of non-Mendelian element	Chromosomal mutant that loses the element	Relation	Does replacing the chromosomal mutant gene restore the phenotype?
M dsRNA	Killer +	Killer –	Opposite	No
mitDNA	Glycerol +	Glycerol –	Opposite	No
mitDNA-DI	Glycerol –	Glycerol –	Same	No
Prion	Defective	Defective	Same	Yes
[URE3]	USA uptake +	USA uptake +	Same	Yes
[PSI]	Suppressor ⇑	Suppressor ⇑	Same	Yes

Since either the presence of the prion or mutation in the gene for the protein result in deficiency of the normal protein, any phenotype resulting from the absence of this normal protein will be similar in these two conditions. The chromosomal gene for the protein is also required for propagation of the prion. Usually, the presence of a non-chromosomal genetic element produces the phenotype opposite that of mutation of a chromosomal gene needed for its propagation. One exception is a mutant of the non-chromosomal nucleic acid replicon that makes its presence known by elimination of its normal parent (like a defective - interfering [DI] virus). This can be distinguished from a prion by replacing the chromosomal maintenance gene, which will restore the phenotype to normal, in the case of the prion, but not in the case of the mutant nucleic acid replicon.

its negative regulation of the activity of Gln3p, a positive transcription regulator necessary for transcription of many of the genes regulated by this system (**Fig. 2**; ***26–28***).

Recently, we have found evidence that the ammonia signal is transmitted to Ure2p by Mks1p (**Fig. 2**; ***29***). Ure2p is present in the cytoplasm of cells grown on either a rich or poor nitrogen source ***(29)***. Ure2p and Gln3p form a cytoplasmic complex ***(30)***. There is also evidence that the Ure2p–Gln3p pathway is regulated by the target of rapamycin (TOR) kinases, two protein kinases whose activity is inhibited by the antineoplastic drug, rapamycin ***(30–32)***. Rapamycin treatment, by affecting the TOR kinases, affects the phosphorylation of Gln3p, thereby dissociating the Ure2p–Gln3p complex, and allowing Gln3p to enter the nucleus where it can promote transcription of many genes for using poor nitrogen sources ***(30)***. Ure2p itself is phosphorylated in a TOR-dependent reaction in response to rapamycin treatment ***(31,32)***. It remains unclear whether the TOR pathway mediates the turn-on of nitrogen assimilation genes induced by poor nitrogen sources, or if this is a different signal transduction pathway that feeds into Ure2p and Gln3p.

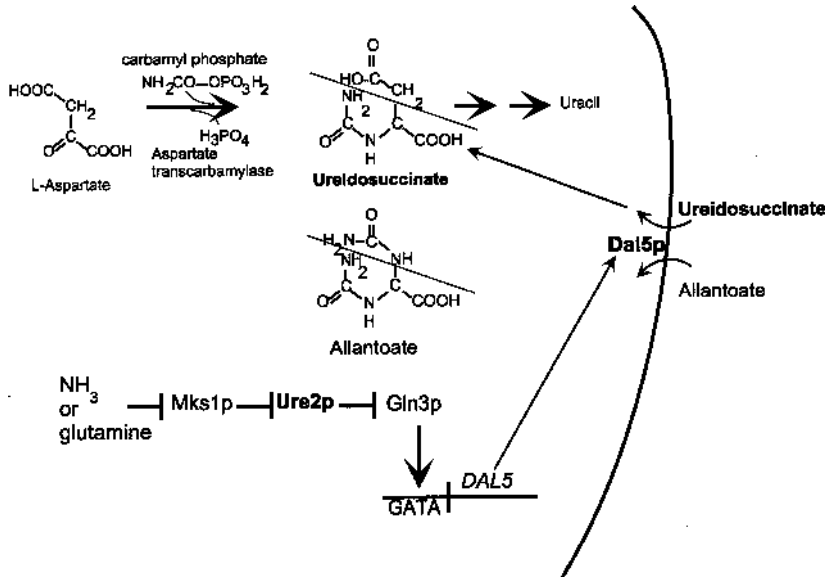

Fig. 2. Ure2p and regulation of nitrogen catabolism. Ure2p receives a signal of abundant ammonia supplies through Mks1p, and passes this signal to Gln3p, preventing it from activating transcription of genes encoding proteins for utilization of poor nitrogen sources.

5. [PSI], a Nonchromosomal Genetic Element Affecting Translation Termination

In 1965, Cox discovered a nonchromosomal genetic element that increased the efficiency with which a weak tRNA suppressor, called *SUQ5*, allowed readthrough of a nonsense mutation (UAA, the "ochre" termination codon) in the *ade2* gene ***(33)***. He named this nonchromosomal element [PSI], perhaps in preparation for naming mutants unable to propagate (PSI) as *PNM* for "PSI no more" ***(34)***. Efforts to identify [PSI] with any of the known nonchromosomal replicons were unsuccessful ***(35,36)***; reviewed in **ref.** ***37***, and the molecular nature of both [PSI] and [URE3] were long-standing mysteries in the yeast world.

In apparently unrelated work, omnipotent suppressor mutations, recessive chromosomal mutations resulting in elevated readthrough of nonsense mutations, were found to define two genes, *SUP35* and *SUP45* ***(38,39)***. Recent studies have shown that Sup35p and Sup45p are the subunits of the translation release factor that recognizes the termination codon on the mRNA, and releases the completed peptide from the last tRNA (**Fig. 3**; ***40,41***). Suppressor tRNAs have a mutated anticodon that recognizes a termination codon and results in insertion of an amino acid, instead of chain termination. This type of suppres-

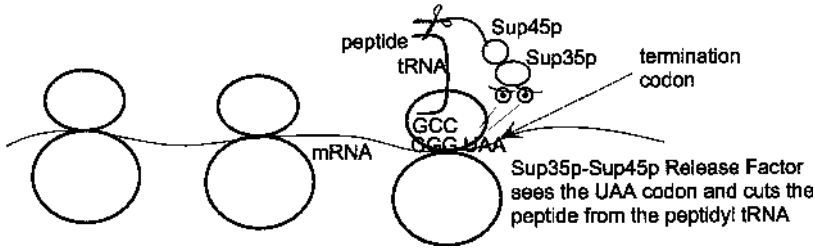

Fig. 3. Sup35p is a subunit of the translation termination factor. Mutant tRNAs that read termination codons (suppressor tRNAs) compete with the normal translation termination factor. Therefore, mutants in *sup35*, or an abnormal (prion) form of Sup35p, should allow suppressor tRNAs to be more efficient.

sion event is always in competition with the translation release factor, so *sup35* or *sup45* mutations are expected to increase the frequency of suppression by suppressor tRNAs.

6. [URE3] Satisfies the Genetic Criteria for a Prion of Ure2p

Growing cells in the presence of millimolar concentrations of guanidine results in efficient curing of [URE3] ***(7,37)***. When cells are cured in this manner, one may isolated derivatives that have again become [URE3] ***(7)***. Thus, [URE3] is reversibly curable. Overproduction of Ure2p increases by 20–200-fold, the frequency with which [URE3] arises *de novo* ***(7)***. Finally, the *ure2* mutants and [URE3] strains have the same phenotype, and *ure2* mutants are unable to propagate the [URE3] nonchromosomal genetic element ***(7,25)***. Thus, [URE3] satisfies all three genetic criteria as a prion of Ure2p.

7. [PSI] Satisifies the Genetic Criteria for a Prion of Sup35p

Growth of [PSI]-containing cells in high-osmotic-strength media results in efficient curing ***(42)***. From cells cured in this manner can again be isolated cells that have acquired [PSI] ***(43)***. The frequency of *de novo* appearance of [PSI] is increased 100-fold by the overproduction of Sup35p ***(44)***. Finally, the phenotype of *sup35* mutants and PSI strains is essentially the same, and *sup35* mutants are unable to propagate PSI ***(45,46)***. Thus, PSI satisfies the three genetic criteria as a prion of Sup35p. The clear parallel between the evidence in these two systems suggested that they were both prions particularly convincingly ***(7)***. These three characteristics are each evidence not only against a nucleic acid replicon, but also evidence specifically for a prion. Various other epigenetic phenomena are known to show the equivalent of reversible curability, but none of those are nonchromosomal genetic elements.

8. Prion Domains of Ure2p AND Sup35p Are Rich in Asparagine and Glutamine

Deletion analysis of the *URE2* gene showed that the N-terminal 65 amino acid residues were sufficient to induce [URE3] formation ***(47)***. The same region could stably propagate [URE3] in the complete absence of the C-terminal part of the molecule ***(48)***. More detailed examination showed that extending this region to residues 1–80 increased the efficiency of prion induction ***(49)***. This region is rich in asparagine residues, with several runs, and is relatively rich in serine and threonine as well (**Fig. 4**). Deletion of any of the asparagine-rich regions dramatically reduced the prion-inducing activity of the remaining protein ***(49)***.

The C-terminal part of Ure2p is sufficient to perform its nitrogen regulation function ***(28,47)***, although this function is mildly impaired unless the protein is either overproduced or includes the N-terminal prion domain. The C-terminal nitrogen regulation domain is not functionally affected by [URE3], unless the prion domain is covalently attached ***(48)***. Deletions in parts of the C-terminal domain result in a dramatic 100-fold increase in the efficiency with which the remainder of the molecule induces [URE3] ***(47)***, suggesting that the C-terminal part of Ure2p stabilizes the prion domain, preventing it from undergoing the prion change.

Although the predominant role in [URE3] prion generation and propagation is played by the N-terminal 80 residues of Ure2p, deletion of residues 221–227 within the C-terminal region did not affect nitrogen regulation activity, but did eliminate prion-inducing activity of the otherwise intact overproduced Ure2p ***(49)***. This region has no Asn or Gln residues, and is, of course, dispensable for prion-inducing activity by Ure2 fragments with further deletions.

A further indication of the complexity of prion-inducing activity is the finding that two nonoverlapping fragments of Ure2p are each capable of inducing the high-frequency appearance of [URE3] ***(49)***. Although a fragment lacking the first defined prion domain (residues 1–65) is inactive in prion induction, further deletion of the prion-inhibiting regions 151–157 and 348–354 produces a fragment that is now able to induce [URE3] ***(49)***. If either of the positive segments, 66–80 or 221–227, are deleted from this fragment, this activity is again lost ***(49)***. Clearly, prion-inducing activity is a complex process involving both intramolecular and intermolecular interactions. The prion-promoting and prion-inhibiting activities of regions of Ure2p are summarized in **Figure 4**.

Deletion analysis of Sup35p showed that its N-terminal 123 amino acid residues (the N domain) are critical for prion propagation and generation ***(46,50,51)***. The C-terminal residues, 254–685, are essential for cell growth ***(50)*** and is the region with homology to EF-1α ***(52–54)***. Deletion of this region makes a strain unable to propagate [PSI] ***(46)***, and overproduction of this region induces the *de novo* appearance of [PSI] ***(51)***. Indeed, the frequency of [PSI]

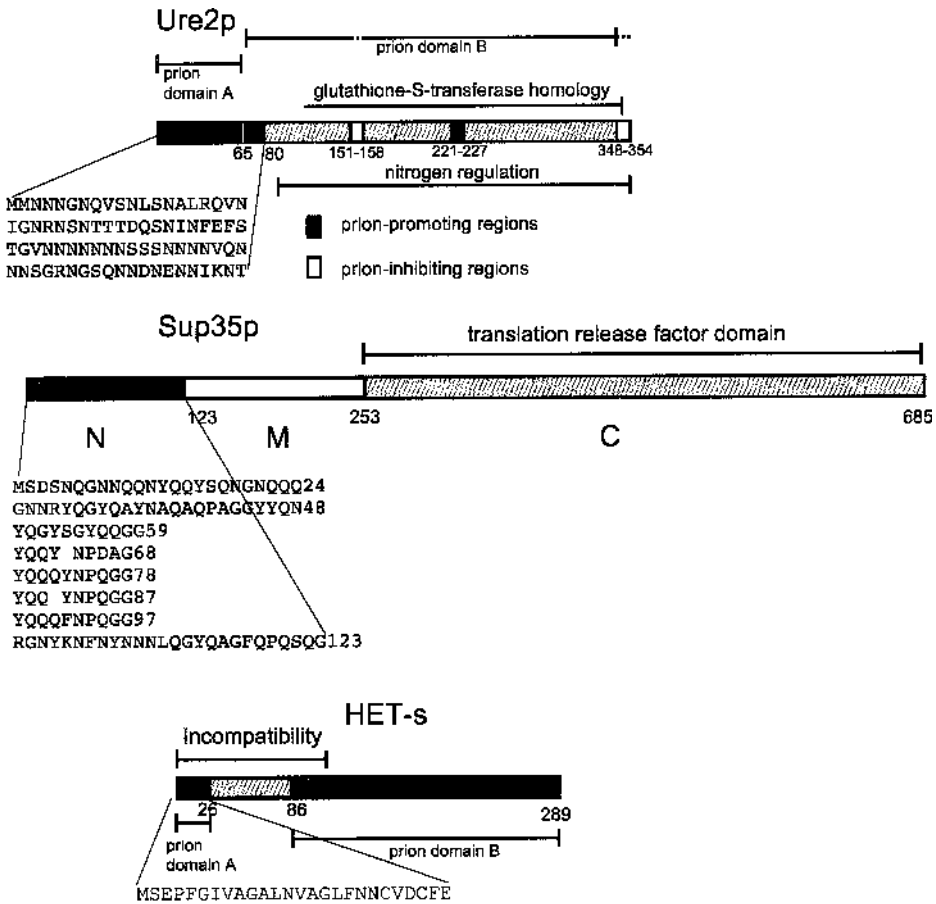

Fig. 4. Prion domains of Ure2p, Sup35p, and HET-s. Prion-promoting and -inhibiting domains are indicated. Two nonoverlapping fragments of either Ure2p or HET-s can induce or propagate the prion forms ***(49,83)***. The prion domains of Sup35p were determined by TerAvanesyan et al. ***(46)***.

prion generation by this prion domain alone is 66-fold greater than that by similar overproduction of the full-length protein, suggesting, as for Ure2p and [URE3] generation, that the C-terminal domain stabilizes the N-terminal prion domain in the nonprion form ***(55)***.

The prion domain of Sup35p, like that of Ure2p, is rich in glutamine and asparagine residues. It also has seven copies of an imperfect repeat sequence, QGGYQQQYNP (**Fig. 4**). Mutagenesis of this region has shown that the glutamines are important for prion generation and propagation, and that the N-terminal part of the prion domain may be replaced with

poly(glutamine), without loss of prion activity ***(34,45,46,56)***, suggesting a relation with the Gln repeats of some triplet-repeat diseases. In addition, the repeat region contributes to prion activity, with deletions reducing activity, and increasing the number of repeats, increasing prion activity ***(46,57)***.

The significance of the repeats of Sup35p, and their parallel to similar repeats in the N-terminal region of PrP, is somewhat uncertain. It is clear that the Sup35p repeat region is involved in prion generation, because it is both necessary for, and its expansion can increase, prion effects. Human mutants, with increased numbers of repeats in PrP, are among those with hereditary Creutzfeldt-Jakob disease ***(58)***, but deletion of the repeat region does not interfere with scrapie propagation in mice ***(59)***.

9. Further Evidence that [URE3] Is a Prion of Ure2p

The prion model for [URE3] predicts that the Ure2 protein should be altered in some way in [URE3] strains, compared to wild-type strains. It was shown that, indeed, Ure2p is more protease-resistant in extracts of [URE3] cells than in extracts of (ure-o) cells (**Fig. 5**; ***47***). This protease resistance is not simply a general concomitant of derepressed nitrogen regulation, but is a special feature of [URE3] strains ***(48)***.

It was important to show that [URE3] is truly arising *de novo*, and is not a mutant form of a nucleic acid replicon already present in the cell. Such a replicon would depend on the same chromosomal genes as does [URE3], but would have inactivated functional genes. Thus, a *ure2* mutant would have lost this replicon, and would no longer be able to give rise to [URE3] derivatives, even if the *URE2* gene were subsequently replaced. In fact, starting with a *ure2*Δ strain, replacing the *URE2* gene on a plasmid made the strain capable of giving rise to [URE3] derivatives, ruling out this model ***(48)***. Spontaneous generation of prions does happen.

It was stated above that overproduction of Ure2p induces the *de novo* appearance of [URE3], and it was critical to show that, in these experiments, it was the protein, not the overproduced *URE2* mRNA or the *URE2* gene itself in high copy number, that was making [URE3] appear. When *URE2* is placed under the control of the inducible *GAL1* promoter, formation of [URE3] is only induced when the promoter is turned on, so that the high copy number of the gene is not the critical factor ***(7)***. Removing a single nucleotide from codon 40 of *URE2*, and introducing a compensatory nucleotide in codon 80, alters the reading frame of most of the prion domain, and produces a protein of normal size with full nitrogen regulation activity, but no prion-inducing activity whatsoever, and normal levels of *URE2* mRNA (**Fig. 6**; ***48***). Replacing the single nucleotide removed in codon 40 raises prion-inducing activity to a high level, without changing the mRNA levels. That it is the reading frame alteration that

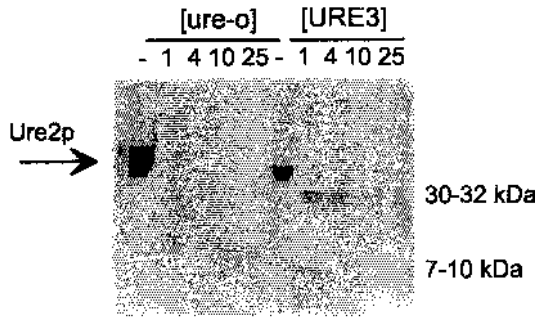

Fig. 5. Protease resistance of Ure2p in (URE3) prion-containing cells. Ure2p is more proteinase K resistant in extracts of (URE3) cells than in extracts of wild-type cells, indicating that Ure2p is altered in (URE3) strains ***(47)***. Furthermore, the pattern of protease-resistant fragments is similar to that produced from the amyloid form of Ure2p produced in vitro ***(66)***. The figure shows a Western blot of extracts probed with antibody to the prion domain of Ure2p.

is critical here, and not this particular base in codon 40, is shown by lack of effect of removing the entire codon 40 from the full-length gene or from the prion domain alone ***(48,49)***. This proves that it is the Ure2 protein whose overproduction results in the *de novo* formation of the [URE3] inheritable element.

Positive regulatory circuits can produce a nonchromosomal genetic element, and Ure2p is a regulator of transcription (via its effects on Gln3p). However, it is clear that this is not the mechanism of [URE3]. The prion domains and nitrogen regulation domains of Ure2p are separable (**Fig. 4**; ***47***) , and the propagation of [URE3] does not depend on the repressed or derepressed state of nitrogen catabolism ***(48)***.

10. Further Evidence that [PSI] Is a Prion of Sup35p

A nonsense mutation in the prion domain of *SUP35* has little effect on mRNA levels, but eliminates the prion-inducing activity of the overexpressed gene, indicating that it is the protein, not the mRNA, that is inducing [PSI]s appearance ***(51)***.

One nonprion model for [PSI] was a self-propagating suppression, in which expression of a suppression-promoting protein itself required suppression. This model is ruled out by the lack of correlation of suppression level with propagation of [PSI] ***(46)***. Expression of the Sup35p prion domain as a separate molecule from the C-terminal domain allows propagation of [PSI] in an antisuppressing environment.

The Sup35 protein was found to be aggregated specifically in extracts of [PSI+] strains ***(60)***, an aggregation reflecting in vivo aggregation of the protein, as shown by using a Sup35–GFP fusion protein ***(61)***.

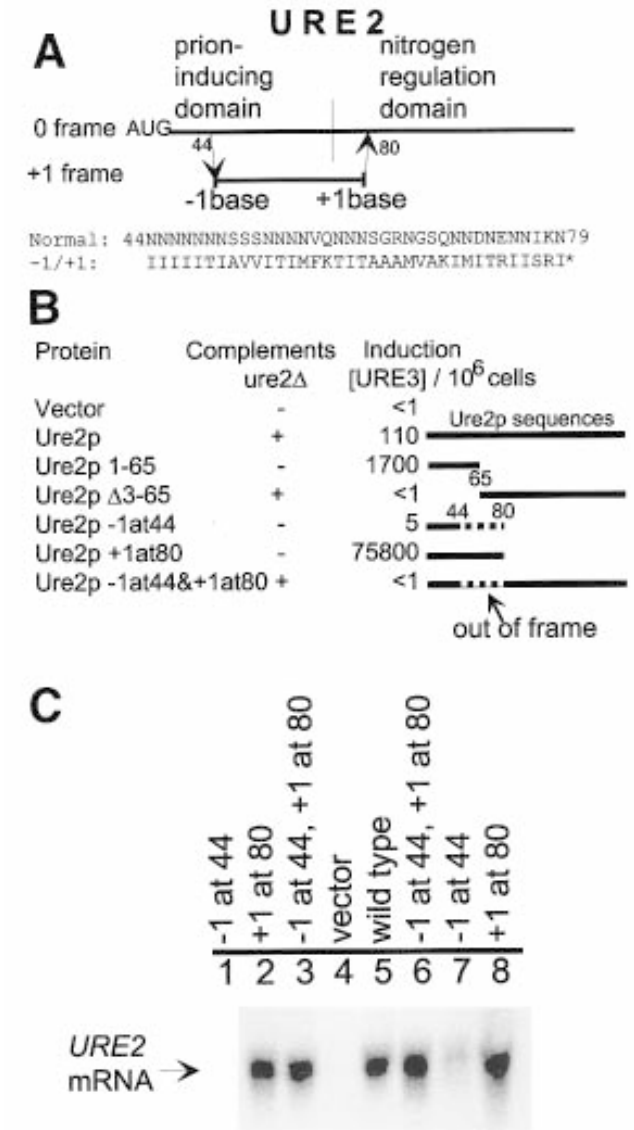

Fig. 6. The Ure2 protein induces *de novo* [URE3] formation. Overproduction of Ure2p from an inducible *GAL1* promoter results in an increased frequency with which [URE3] arises ***(7)***. Adding one nucleotide in codon 80 of *URE2* produces a prion domain fragment with greatly elevated [URE3]-inducing activity ***(48)***. Further removing one nucleotide in codon 44 changes the reading frame of most of the prion domain, eliminating [URE3]-inducing activity, without changing the level of mRNA ***(48,54)***. It was confirmed that the nucleotide removed from codon 44 was not of special importance, by removing the entire codon 44; this did not affect prion-inducing activity ***(49)***.

11. Self-Promoting Filament Formation by Sup35p

The genetic properties of [PSI] and *SUP35* indicate that [PSI] is a prion form of Sup35p, but do not indicate what is the mechanism of the prion. Several biochemical studies suggest that [PSI] is an amyloid form of Sup35p whose self-catalysis of amyloid formation is the basis of propagation of the [PSI] prion.

The aggregates of Sup35p, found specifically in extracts of (PSI+) strains, were found to be self-propagating ***(62)***. Addition of [PSI+] extracts, containing aggregated Sup35p, to extracts of [psi-] cells, whose Sup35p was mostly soluble, resulted in the conversion of the soluble Sup35p to the aggregated form ***(62)***.

In a different approach, King et al. ***(63)*** showed that $Sup35p^{2–114}$ made in *Escherichia coli*, spontaneously formed filaments in 40% acetonitrile, at pH 2.0. These filaments were high in β-sheet structure, had slight protease resistance, and showed clear birefringence on staining with Congo red, a staining property characteristic of amyloid.

Glover et al. ***(64)*** made full-length Sup35p, as well as several fragments, in *E. coli*, and found that all fragments containing the prion domain spontaneously formed filaments in vitro. The full-length Sup35p formed filaments after a long delay, which could be shortened by addition of either preformed filaments or by addition of an extract of a [PSI+] strain. All of the filaments formed were high in β-sheet content, and those formed from the prion domain and middle domain showed birefringence on staining with Congo red (S. Lindquist, personal communication).

12. Amyloid Formation by Ure2p Promoted by the Prion Domain of Ure2p

The genetic studies described above indicate that [URE3] is a prion of Ure2p, a self-propagating inactive altered form of the Ure2 protein. The first hint of the mechanism by which [URE3] is self-propagating was the finding that Ure2p is relatively proteinase K-resistant in extracts of strains carrying [URE3], compared to wild-type strains ***(47)***. The Ure2p in extracts of wild-type strains was rapidly digested, but Ure2p in extracts of [URE3] cells gave rise to transient 30 and 32 kDa species, and to a longer-lived 7–10 kDa species (**Fig. 5**). The specificity of the antibody used in the Western blots done for this experiment indicated that the 7–10 kDa species is mostly derived from the N-terminal prion domain of Ure2p ***(47)***.

Using Ure2-GFP fusion genes, it was found that Ure2p is aggregated specifically in [URE3] cells (**Fig. 7**; ***65***). This in vivo aggregation required the prion domain of Ure2p, as well as the presence of the [URE3] prion. In [ure-o] cells, the Ure2-GFP fusion protein was evenly distributed in the cytoplasm ***(65)***.

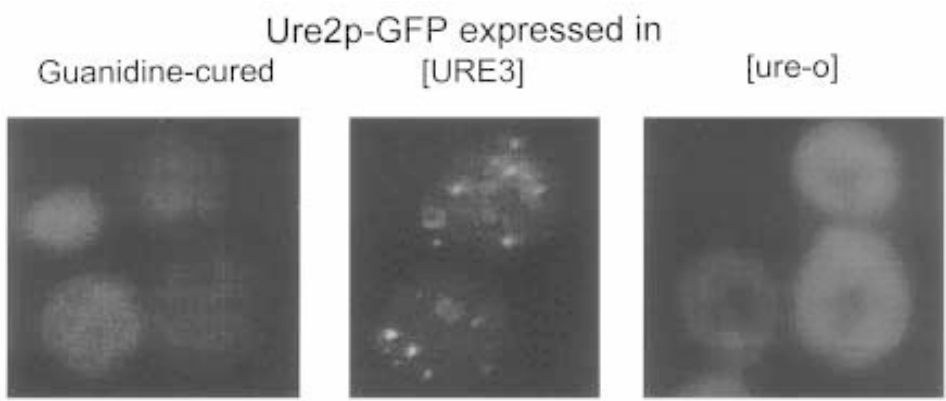

Fig. 7. Aggregation of Ure2p in [URE3] cells. A Ure2p-green fluorescent protein fusion protein, which was both active in nitrogen regulation and capable of involvement in the prion change, was expressed in wild-type, [URE3] prion-containing, and cured cells. In normal cells, the fusion protein was evenly distributed throughout the cytoplasm, but in prion-containing cells it was aggregated ***(65)***.

Taylor et al. ***(66)*** found that synthetic $Ure2p^{1–65}$ spontaneously formed amyloid filaments in vitro (**Fig. 8**). These 45Å filaments had all of the characteristics of classic amyloid: They were filaments that were high in β-sheet content, protease-resistant, and showed the birefringence, on staining with Congo red, characteristic of amyloid (**Fig. 9**; ***66***). Moreover, these filaments formed at physiological pH and salt conditions.

Under conditions in which the full-length native Ure2p purified from yeast is stably soluble, addition of equimolar amounts of the $Ure2p^{1–65}$ peptide leads to the formation of cofilaments 200Å in diameter, consisting of equimolar amounts of the $Ure2p^{1–65}$ peptide and full-length Ure2p. This cofilament formation is highly specific, in that $Ure2p^{1–65}$ does not form cofilaments with proteins other than Ure2p. Moreover, $A\beta^{1–42}$, which also forms amyloid in vitro, does not form cofilaments with Ure2p ***(66)***. These cofilaments also have all the properties of amyloid. Their protease resistance shows a pattern similar to that seen in extracts of [URE3] strains, with transiently stable 30 and 32 kDa species and a stable 7–10 kDa species that reacts on Western blots with antibody specific for the prion domain of Ure2p. Adding a small amount of cofilaments to an excess of native soluble Ure2p induces the conversion of most of the Ure2p to amyloid ***(66)***.

These results indicate that amyloid formation is the basis for the [URE3] phenomenon. Amyloid formation in vitro is promoted by the same part of the molecule (the prion domain) that is responsible for prion formation in vivo; the

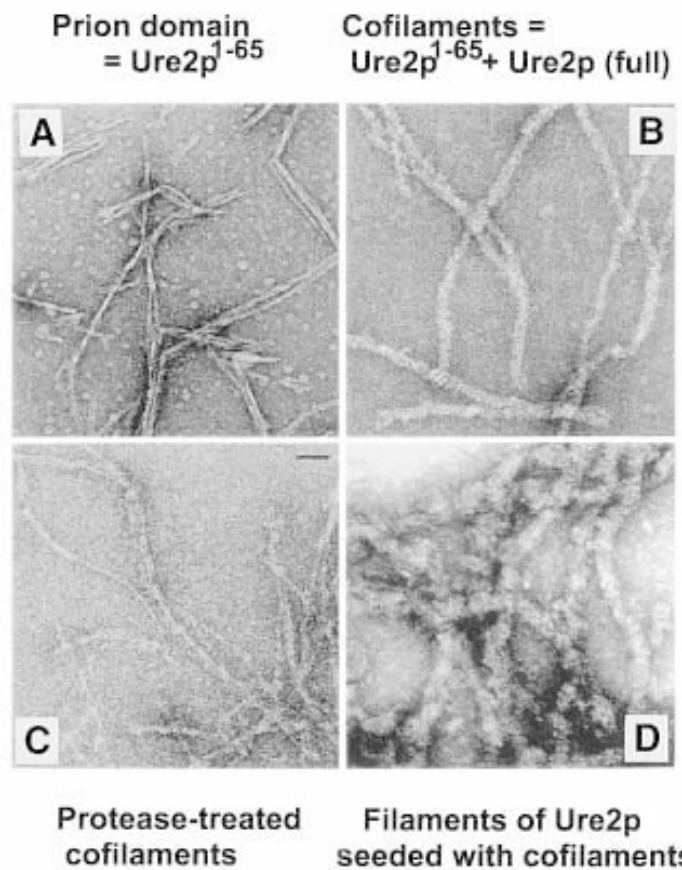

Fig. 8. Amyloid fiber formation by native Ure2p directed by the Ure2p$^{1–65}$ prion domain. Chemically synthesized Ure2p$^{1–65}$ spontaneously forms 45 Å diameter amyloid filaments in vitro **(A)** ***(66)***. Mixing equimolar amounts of synthetic Ure2p$^{1–65}$ with native soluble full length Ure2p produces 200 Å diameter cofilaments **(B)** which, on digestion with proteinase K, yields core-resistant filaments composed mostly of the prion domain of Ure2p **(C)** ***(66)***. Addition of small amounts of cofilaments (**B**), to excess soluble native full length Ure2p, results in seeding of polymerization of Ure2p forming 400 Å amyloid filaments **(D)** ***(66)***.

pattern of Ure2p protease resistance is similar for extracts of [URE3] strains and amyloid formed in vitro; Ure2p is aggregated specifically in [URE3] strains. It remains to be shown that Ure2p amyloid is in fact present in [URE3] strains.

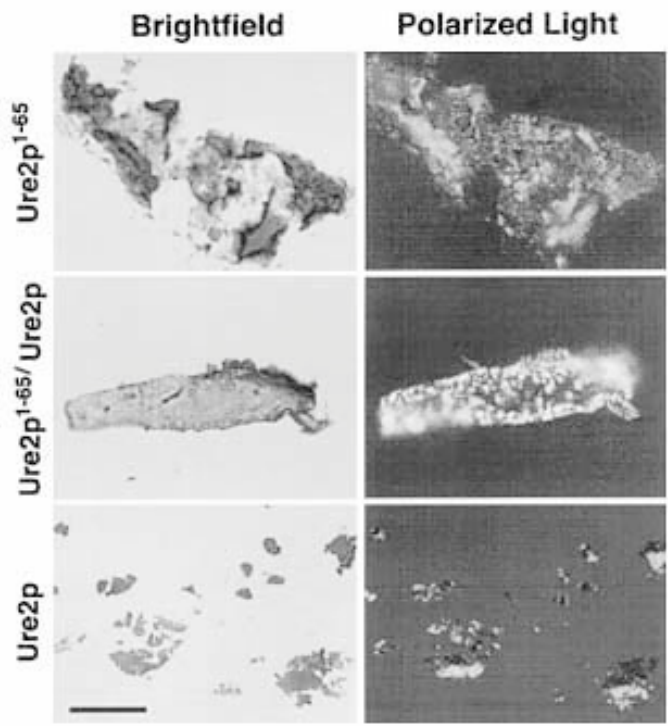

Fig. 9. Birefringence of Ure2p amyloid filaments on staining with Congo red.

13. Role of Chaperones in Prion Propagation

One dramatic dividend from the discovery of yeast prions has been the finding by Chernoff et al. that chaperones have a critical impact on prion propagation ***(67,68)***. Heat shock protein 104 (Hsp104) is a chaperone of yeast that is capable of solubilizing proteins that have aggregated as a result of exposure to high temperature ***(69)***. It is the only heat-shock protein in yeast that is essential for surviving heat shock, and not for growth within the usual temperature range ***(70)***.

Overexpression of Hsp104 results in loss of [PSI+], accompanied by disappearance of the Sup35p aggregates ***(67,68)***. This effect is probably explained by the protein-disaggregating ability of Hsp104. Deletion of the *HSP104* gene also results in loss of [PSI+] ***(68)***. Thus, Hsp104 levels must be optimal for [PSI] propagation. Overproduction must disaggregate the Sup35p amyloid, but why does underproduced Hsp104 result in loss of [PSI]? Two models have been proposed. One posits that Hsp104 is necessary for the generation of an intermediate form of Sup35p, which can then be converted to the [PSI+] form ***(68)***. Another model explains the requirement for Hsp104 by assuming that, without Hsp104, the aggregation would proceed to the extreme of forming just

one lump in the cell, which would then be segregated to only one of the daughter cells; the other is, in effect, cured of [PSI+] ***(60)***. It is suggested that normal levels of Hsp104 break up the Sup35p amyloid into smaller lumps, thus assuring each daughter cell of receiving one or more fragments of amyloid. The fact that Sup35p amyloid formation proceeds well in vitro, in the absence of Hsp104 ***(63)***, argues against the first model, but further work will be needed to resolve this issue.

Hsp70s are a class of chaperones that assist protein folding and block aggregation or denaturation, by binding to hydrophobic patches on the surfaces of partially folded (or partially unfolded) proteins (reviewed in **refs**. ***71*** and ***72***). The curing of [PSI+] by overproduction of Hsp104 is partially blocked by the simultaneous overproduction of Ssa1p, one of the family of yeast Hsp70s ***(73)***. Another family of Hsp70 proteins, the Ssb family, functions in assisting the folding of newly synthesized proteins ***(74)***. The Ssb proteins appear to have effects opposite those of the Ssa proteins. Overproduction of Ssb proteins slightly increases, and elimination of Ssbs significantly decreases, the efficiency of curing of [PSI+] by overproduction of Hsp104 ***(75)***. Neither effect is mediated by an effect on the levels of the Hsp104 protein ***(75)***. These results reflect the complex interactions among heat shock proteins, and, in some cases, may be caused by the regulatory effects of one heat shock protein on others, either directly or by producing a state of stress in the cell. In any case, it is clear from the work of Chernoff et al. ***(75)*** that chaperones play central roles in the generation and propagation of prions.

14. Role of Interacting Proteins in [URE3] and [PSI] Generation

Recent evidence indicates that Mks1p is involved in the nitrogen regulation pathway, communicating the presence of ammonia in the medium to Ure2p and inhibiting Ure2p action on Gln3p (**Fig. 2**; ***29***). We find that deletion of *MKS1* prevents the *de novo* generation of [URE3], even when Ure2p or one of its fragments is overproduced (Edskes and Wickner, in preparation). Although the *mks1*Δ strains are deficient in generation of new [URE3] elements, they are not defective in propagation of [URE3] introduced into the cell by cytoplasmic mixing. [URE3] in such cells is essentially as stable as in wild-type cells, and is similarly curable by guanidine. The inability to generate a new [URE3] is not the result of failure to overexpress Ure2p, nor is there evidence for an altered pattern of degradation of Ure2p. It will be of interest to determine whether Ure2p interacts directly with Mks1p. These results suggest that interaction of Ure2p with Mks1p makes it more suitable for conversion to the prion form (Edskes and Wickner, in preparation).

MKS1 was first identified as a gene negatively regulating growth, and is itself inhibited by the *RAS*–cyclic adenosine monophosphate (cAMP) system

(76). We found that a constitutively active allele of Ras2p also resulted in dramatically reduced generation of [URE3] on overproduction of Ure2p, consistent with the earlier results (Edskes and Wickner, in preparation). *MKS1* was independently identified as *LYS80*, an inhibitor of lysine biosynthesis *(77)*. It is not yet clear how this phenotype relates to the role of Mks1p in nitrogen regulation and prion generation.

These results indicate that cellular regulatory mechanisms can dramatically affect the *de novo* formation of a prion. With further understanding of the control pathways operating on Ure2p as part of the nitrogen regulation system, it is likely that other factors influencing prion generation will be found.

In normal cells, the Sup35 protein is known to be present as a soluble heterodimer with Sup45p, and this heterodimer is responsible for recognition of translation termination codons and release of the completed peptide. Derkatch et al. have found that overproduction of the Sup45 protein blocks the induction of the *de novo* formation of [PSI+] *(78)*. This result indicates that it is the free form of Sup35p that is most capable of undergoing the prion change, and that, in its complex with Sup45p, it is stabilized *(78)*.

The inhibition of prion formation by Sup35p on overproduction of Sup45p is the reverse of the inhibition of prion formation by elimination of Mks1p. The proteins interacting with HET-s or PrP are not yet known, but it is tempting to speculate that they too will affect prion generation.

15. [PIN+], A Non-Mendelian Genetic Element Controls Generation of [PSI+]

Derkatch et al. *(79)* have found that strains differ in their ability to give rise to [PSI+], even when Sup35p is overexpressed. Genetic analysis shows that the ability to give rise to [PSI+] in this way requires the presence of a dominant nonchromosomal genetic element called [PIN+] (for [PSI+] induction) *(79)*. [PIN+] does not require the N-terminal part of Sup35p for its propagation, but whether it requires the C-terminal part of Sup35p cannot be tested.

Although overproduction of Hsp104 cures [PSI+] efficiently, it does not cure [PIN+] *(79)*. In contrast, guanidine cures both [PSI+] and [PIN+] *(79)*. This suggests that guanidine does not cure simply by inducing Hsp104 production.

Although [pin–] strains cannot be induced to generate [PSI+] by overproduction of intact Sup35p, they are fully capable of becoming [PSI+] by overproducing the Sup35p prion domain *(79)*. Derkatch et al. *(79)* suspect that [PIN+] is a prion, but demonstration of this idea will be difficult, until a candidate protein is found whose overproduction induces [PIN+], and which is necessary for [PIN+] propagation.

16. [Het-s], A Prion of *Podospora* Required for a Normal Function

16.1. Sexual Mating vs *Hyphal Anastomosis*

Most strains of *Saccharomyces cerevisiae* are diploid, and the starvation conditions that initiate the meiosis–sporulation pathway are commonplace in nature. The haploid mating-competent products of meiosis quickly find a compatible partner, and mating reconstitutes the diploid state. This sexual mating seems designed to generate diversity in the progeny, so that some may survive uncertain future environmental conditions.

Filamentous fungi have two kinds of mating. Sexual mating, like that of *S. cerevisiae* (and higher organisms), is designed to produce diverse offspring, and so it requires that parents differ at one or more special loci (mating-type loci). Hyphal anastomosis is another form of mating, in which two fungal colonies fuse when they meet, probably in order to share nutrients. In yeast and other fungi, infectious entities, such as viruses, pass from cell to cell exclusively via cell-to-cell contact, such as occurs in mating and hyphal anastomosis. This poses a risk, which is essentially a sexually transmitted disease of fungi. In filamentous fungi, the sexual spores are usually devoid of viruses, perhaps because these cells have little cytoplasm. However, hyphal anastomosis is accompanied by the free flow of cytoplasm, and even nuclei, from one colony to another. Perhaps for this reason, fungi limit hyphal anastomosis to partners that pass a test for genetic identity, the presumption being that genetically identical partners already carry the same viruses. In *Podospora anserina,* this test is identity at nine chromosomal loci, called *het* loci (reviewed in **ref.** ***80***). Partners with different alleles at even one of the *het* loci will attempt to fuse hyphae, but quickly recognize that they were not "meant for each other," and form a barrier between the colonies that prevents further fusion attempts.

One of these loci, called *het-s*, has two alleles, *het-s* and *het-S*. The *het-s* locus is unusual, in that it can have either of two phenotypes, and this difference in phenotypes is controlled by a nonchromosomal genetic element, called [Het-s] ***(81)***. Colonies that are *het-s* and have [Het-s] show the usual reaction of incompatibility when they meet a colony that is *het-S*. However, *het-s* colonies that do not have the [Het-s] nonchromosomal genetic element are able to fuse equally with *het-s* or *het-S* partners (a neutral phenotype) (**Fig. 10**).

16.2. [Het-s] Has Properties of a Prion of the HET-s Protein

The [Het-s] nonchromosomal genetic element spreads from one colony to another by hyphal anastomosis. However, [Het-s] is apparently not included in sexual spores, and so it is lost from the meiotic offspring of a sexual cross ***(82)***. This is a form of natural curing. In a colony arising from a meiotic offspring lacking [Het-s], this element often arises again, and spreads through the colony

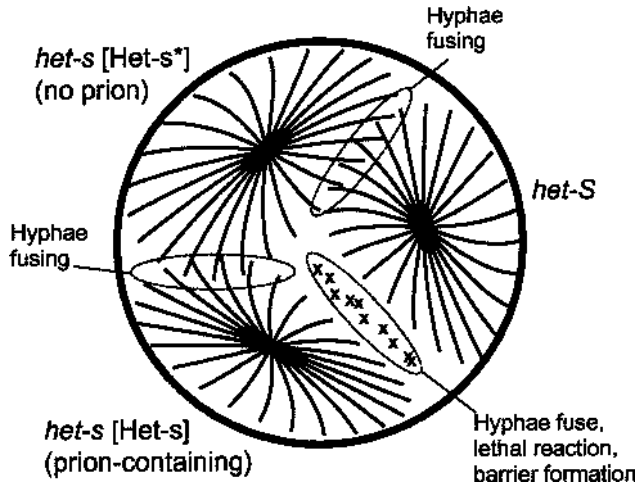

Fig. 10. [Het-s] is a prion necessary for heterokaryon incompatibility, a normal fungal function. Two colonies of the filamentous fungus *Podospora* will fuse to form heterokaryons when their hyphae meet, if they are genetically identical at 9 chromosomal loci, called *het* loci. Heterokaryon incompatibility results from different alleles at any of these loci, and the colonies do not fuse. One such locus, called *het-s*, with alleles *het-s* and *het-S*, produces heterokaryon incompability, only if the protein product of the *het-s* allele is in a prion form ***(8)***. [Het-s*] denotes the absence of [Het-s].

(82). This is reminiscent of the reversible curing criteria for yeast prions ***(7)***. Coustou et al. ***(8)*** have found that overproduction of the protein encoded by *het-s* increases the frequency with which the [Het-s] element arises, and the *het-s* gene is necessary for the propagation of the [Het-s] non-chromosomal genetic element. They therefore proposed that [Het-s] is a prion form of the HET-s protein, a suggestion that they support by showing, further, that the *het-s* protein is relatively protease-resistant in extracts of a [Het-s] strain, compared to a strain lacking [Het-s].

16.3. The HET-s Minimal Prion Domain Is the N-terminal 26 Amino Acids

Deletion mutants of *het-s* were tested for their ability to propagate [Het-s], and to show the incompatibility reaction ***(83)***. It was found that expression of residues 1–26 was sufficient to allow propagation of the [Het-s] prion; to express the incompatibility reaction required residues 1–112 (**Fig. 4**). Residues 86–289 were also sufficient to propagate the [Het-s] prion. Thus, just as Ure2p has two nonoverlapping regions, either of which is sufficient, when overexpressed, to induce the *de novo* appearance of [URE3], the protein encoded by *het-s* has two nonoverlapping regions, either of which can propagate the [Het-s] prion ***(83)***.

Expressing both the HET-s and HET-S alleles in the same cell results in a sublethal phenotype, presumably because of the formation of toxic complexes of the two proteins ***(83)***. This allowed selection of mutants of the *het-s* or *het-S* genes, which could no longer give this phenotype, or which could not propagate [Het-s]. The same selection also produced mutants in chromosomal genes affecting prion propagation, whose analysis will doubtless be of considerable interest.

17. Curing of Yeast Prions and Blocking Yeast Prion Generation: Can These Methods Be Used in Mammals?

The first curing method for any prion was discovered by Singh et al. in 1979, who found that growth, or just incubation, of cells in hypertonic medium resulted in high frequency loss of [PSI+] ***(42)***. Curing was found using either 2.5 *M* KCl or 1.75 *M* ethylene glycol. A survey of other chemicals for curing activity showed that growth in the presence of millimolar concentrations of guanidine HCl are efficient cures of [PSI+] ***(84)***. The mechanism of curing by guanidine is as yet unknown, but some parameters of its action have been determined. Exposure of [PSI+] cells to curing doses of guanidine do not result in immediate loss of the prion. Four generations of growth are required before cured cells begin to appear ***(85)***. Incubation of cells, in stationary phase, in the presence of guanidine does not cure, nor does heat or ethanol stress enhance curing.

Similar concentrations of guanidine also cure [URE3] ***(7,37)***. [URE3] can also be cured by overexpression of Ure2p–GFP fusion proteins, or by overexpression of certain fragments of Ure2p ***(65)***. It has been suggested that these fusion proteins or fragments bind to the amyloid growing filaments in such a way that they block further propagation of the filament (**Fig. 11**).

18. Notes

[URE3] requires Hsp104p for propagation and is cured by overexpressed Ydj1p. Deletion or mutation of *HSP104* results in loss of [URE3] ***(93)***, as was previously shown for [PSI] ***(67,68)***. However, while [PSI] is also cured by overexpression of Hsp104p, [URE3] is not. Overexpression of the Hsp40 family chaperone Ydj1p cures [URE3] ***(93)***, but does not cure [PSI] (Dan Masison, personal communication). Differences in response to the state of cellular chaperones may reflect differences in the structure of the Ure2p and Sup35p aggregates.

Evidence for Ure2p amyloid in [URE3] cells. While both Sup35p and Ure2p can form amyloid in vitro, the state of neither protein in prion-containing cells is known. Recently, electron microscopic examination of thin sections of [URE3] cells overproducing Ure2p has revealed the presence of networks of filaments in the cytoplasm ***(96)***. These filaments were shown by

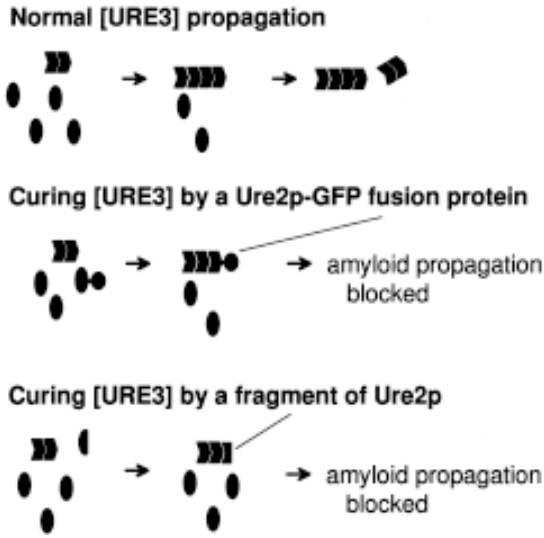

Fig. 11. Curing of the [URE3] prion by fusion proteins or fragments of Ure2p ***(65)***.

Table 2
Comparison of Evidence for the Prion Etiology of Scrapie, [URE3], [PSI], and [Het-s]

Evidence	Scrapie	[URE3]	[PSI]	[Het-s]
Reversible curing	No	Yes	Yes	Yes
Protein overproduction → infectious element	No	Yes	Yes	Yes
Phenotype relation	No	Yes	Yes	No
Spontaneous generation caused by protein	No	Yes	Yes	No
In vitro amyloid propagation	Yes	Yes	Yes	No
Altered protein in infected cells	Yes	Yes	Yes	Yes
UV resistance of infectious agent	Yes	No	No	No
Purification of infectious agent	Yes	No	No	No

immuno electron microscopy to be composed of Ure2p. No filaments were seen in cells lacking [URE3]. Antibody to the C-terminal domain of Ure2p readily detected the filaments while antibodies specific to the N-terminal were less able to do so. Examination of extracts of these cells suggests an explanation for this result. A substantial fraction of the Ure2p in extracts of [URE3] cells, perhaps 3/4 of the total, is insoluble in boiling 3M urea-20% SDS, a property typical of many amyloids. The portion that can be solubilized under these conditions is protease-resistant, as shown previously. The urea-SDS insoluble material reacts with antibody to the C-terminal domain of Ure2p, but not to the N-terminal domain, suggesting a structure in which the prion domain

forms a compact inaccessable structure of stacked beta sheets to which is attached the peripheral C-terminal domain ***(96)***.

Ure2C structure closely resembles glutathione-S-transferases. Two groups have recently reported the structure of the C-terminal domain of Ure2p and found that, consistent with the known amino acid sequence homology with glutathione - S - transferases, the nitrogen regulation domain shows close structural similarity to these enzymes ***(86,97)***. However, structural differences at the active site - homologous region ***(86,97)*** explain why Ure2p has not been found to have GST activity ***(88)***. Two parts of nitrogen regulation domain that stabilize the prion domain ***(47)*** are present in the structure near the presumed location of the N-terminal domain ***(97)***.

Sup35p N-termini from other species can be prion domains. The C-terminal domain of Sup35p from the yeasts *Pichia methanolica, Pichia pastoris, Candida albicans, Kluyveromyces lactis, Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* are closely homologous to that of *S. cerevisiae*. The N-termini are less homologous, but retain the octapeptide repeat pattern and are all rich in asparagine, glutamine and glycine ***(92,94)***. Fusions of the N-termini of these various yeasts to the *cerevisiae* C-terminus were used to test prion domain activity. In one study, the *Pichia pastoris* N-terminal domain was shown to have all of the expected genetic and biochemical properties of a prion ***(91)***. Properties of other fusions are consistent with prion activity, but less completely documented ***(87,94)***. Little or no transmission of the prion state was observed between fusion proteins with N-termini from different species, a result interpreted as the analog of the species barrier seen in the mammalian transmissible spongiform encephalopathies ***(87,91,94)***.

Rnq1p can be a prion. Since the prion domains of Ure2p and Sup35p are both rich in asparagine and glutamine, and these residues are important for prion activity (*see* **Subheading 8.**) candidates for new yeast prions were sought among asparagine-glutamine rich protein sequences. One protein, whose only known feature is that it is Rich in N and Q (Rnq1p) was found aggregated in some strains and soluble in others ***(95)***. Moreover, this aggregation was transmissible by cytoplasmic transfer, curable by guanidine and required Hsp104 for its propagation. Although the presence of aggregation (or indeed deletion of the gene) did not produce a recognized phenotype, this is apparently a prion.

Hsp70s are essential for propagation of [PSI+]. Jung et al. have shown that Hsp70 proteins of the Ssa family are necessary for the propagation of [PSI] ***(89)***. In addition, this group showed that [PSI+] cells have elevated levels of Hsp104, indicating that the presence of [PSI+] is perceived by the cell as a stressful situation ***(89)***. A hint to the mechanism of curing by millimolar concentrations of guanidine comes from the finding that this compound inhibits the in vivo activities of Hsp104 ***(90)***.

19. Conclusions

Yeast and fungal prions have already had an enormous impact on the prion field. The evidence that [URE3] and [PSI+] are indeed prions is, in several ways, stronger than that available for the mammalian systems, thus finally proving the existence of prions (**Table 2**). At the same time, this shows that proteins can be genes. The discovery that chaperones are critical in propagation of (PSI+) has obvious implications beyond yeast. Proteins interacting with Sup35p and Ure2p each determine susceptibility to *de novo* prion generation. The finding that a fungal prion is responsible for a normal cellular function shows that prions need not be a pathological phenomenon, but may in some cases prove to have physiological functions.

We speculate that studies of the cellular factors involved in propagation of yeast prions, and of chemicals or other means capable of curing yeast prions, will suggest treatments useful in human prion or amyloid diseases. Moreover, the use of yeast molecular genetics may also facilitate the discovery of new prions.

20. Summary

We identified the nonchromosomal genes [URE3] and [PSI] of *S. cerevisiae* as prions, based on their special genetic properties, which indicated they were not viruses or plasmids, but were infectious protein forms of Ure2p and Sup35p, respectively. These properties are not yet demonstrated for the TSEs. The [URE3] and [PSI] prions arise *de novo*: they are not mutants of some nucleic acid replicon. The overproduction of the Ure2 and Sup35 proteins, not the mRNAs or the genes, results in 'spontaneous generation' of these prion diseases. The prion domains of Ure2p and Sup35p are rich in Asn and Gln and these residues are critical for prion generation and propagation. The C-terminal domains of Ure2p and Sup35p carry out their cellular functions and stabilize the N-terminal prion domains of each molecule in the normal form.

In [URE3] and [PSI] strains, Ure2p and Sup35p are aggregated, respectively, and Ure2p is protease resistant. Ure2p and Sup35p can form self-propagating amyloid in vitro, and the properties of this amyloid formation indicate that it is the basis of these prion phenomena. The chaperone, Hsp104, is critical in [PSI] propagation, and the Hsp70s are minor modifiers of this effect. Both [PSI] and [URE3] are cured by millimolar concentrations of guanidine in the medium, and [URE3] is also cured by overexpression of various Ure2p fragments, and by fusions of Ure2p with green fluorescent protein. The [Het-s] nonchromosomal gene of the filamentous fungus *P. anserina* is the first example of a prion necessary for a normal cellular function, the heterokaryon incompatibility reaction used by most fungi to avoid the spread of viruses.

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Molecular Pathology of the Prions

Edited by

Harry F. Baker

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It is now widely agreed that the prion protein plays a key role in the molecular pathogenesis of prion diseases—diseases that involve the misfolding of proteins—in both humans and animals. In *Molecular Pathology of the Prions,* noted prion researcher Harry Baker has asked internationally recognized investigators to review the latest developments in, and novel approaches to, understanding the prion protein and prion diseases at the molecular level. Utilizing a variety of cutting-edge techniques, these distinguished scientists seek to define the normal function of a prion protein, to detect and measure the early immune response to prion disease, and to discover possible therapeutic targets. They also use transgenic mice and new electrophysiological investigations to elucidate the pathogenetic mechanisms involved in prion diseases. Other topics addressed include the neuronal death that occurs in prion disease, the different strains of prion disease agents, and the accumulation of protein deposits within brain parenchyma.

State-of-the-art and richly insightful, *Molecular Pathology of the Prions* captures for basic and clinical neuropathologists the latest developments and approaches to understanding the pathogenesis of prion diseases, including research techniques now likely to enjoy broader application for the more common proteinopathies, such as Alzheimer's and Parkinson's diseases.

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