CANADIAN ASSOCIATION OF NEUROSCIENCES REVIEW: Prion Protein and Prion Diseases: The Good and the Bad

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ABSTRACT: In the 1700's a strange new disease affecting sheep was recognized in Europe. The disease later became known as "Scrapie" and was the first of a family of similar diseases affecting a number of species that are now known as the Transmissible Spongiform Encephalopathies (TSEs). The appearance of a new disease in humans linked to the consumption of meat products from infected cattle has stimulated widespread public concern and scientific interest in the prion protein and related diseases. Nearly 300 years after the first report, these diseases still merit the descriptor "strange". This family of diseases is characterized by a unique profile of histological changes, can be transmitted as inherited or acquired diseases, as well as apparent sporadic spontaneous generation of the disease. These diseases are believed by many, to be caused by a unique protein only infectious agent. The "prion protein" (PrP^C), a term first coined by Stanley Prusiner in 1982 is crucial to the development of these diseases, apparently by acting as a substrate for an abnormal disease associated form. However, aside from being critical to the pathogenesis of the disease, the function of PrP^C, which is expressed in all mammals, has defied definitive description. Several roles have been proposed on the basis of *in vitro* studies, however, thus far, *in vivo* confirmation has not been forthcoming. The biological features of PrP^C also seem to be unusual. Numerous mouse models have been generated in an attempt to understand the pathogenesis of these diseases. This review summarizes the current state of histological features, the etiologic agent, the normal metabolism and the function of the prion protein, as well as the limitations of the mouse models.

RÉSUMÉ: La protéine prion et les maladies à prion : le bon et le mauvais. Au dix-huitième siècle, on s'est aperçu que les moutons étaient atteints d'une nouvelle maladie étrange en Europe. Cette maladie a plus tard été appelée « Scrapie » (la tremblante du mouton). C'était la première d'une famille de maladies similaires atteignant un certain nombre d'espèces animales qu'on désigne maintenant sous le vocable d'encéphalopathies spongiformes transmissibles (EST). L'apparition chez l'humain d'une nouvelle maladie liée à la consommation de produits carnés provenant d'animaux infectés a suscité beaucoup d'inquiétude dans la population et d'intérêt scientifique pour la protéine prion et les maladies qui y sont associées. Environ 300 ans après que les premiers cas aient été rapportés, ces maladies méritent toujours d'être décrites comme « étranges ». Cette famille de maladies est caractérisée par un profil unique de changements histologiques, elles peuvent être transmises de façon héréditaires ou être acquises et on peut observer des cas sporadiques dont la génération semble spontanée. Plusieurs croient que ces maladies sont causées par un agent infectieux de nature uniquement protéique. La « protéine prion » (PrPc), une expression utilisée pour la première fois par Stanley Prusiner en 1982, est cruciale pour le développement de ces maladies agissant, semble-t-il, comme substrat à une forme anormale de la protéine associée à la maladie. Cependant, la fonction de PrPc, une protéine qui est exprimée chez tous les mammifères, n'a pas encore pu être décrite définitivement, en dehors du fait qu'elle est cruciale dans la pathogenèse de la maladie. On a proposé plusieurs rôles pour la PrPc sur la base des études in vitro, mais aucun n'a jamais été confirmé in vivo. Les caractéristiques biologiques de la PrPc semblent également inusitées. Plusieurs modèles de souris ont été créés afin d'essayer de comprendre la pathogenèse de ces maladies. Cette revue fait le sommaire des connaissances actuelles sur les caractéristiques histologiques, l'agent étiologique, le métabolisme normal et la fonction de la protéine prion, de même que les limites des modèles chez la souris.

The prototypic prion disease is a naturally occurring disease of sheep and goats called "scrapie" that was first described in the literature in 1732.[1] The prion diseases have since come to be known as transmissible spongiform encephalopathies (TSE) and have been reported in a large number of animal species. These other diseases include, transmissible mink encephalopathy,[2] chronic wasting disease of mule deer and elk,[3] bovine spongiform encephalopathy (BSE),[4] and feline spongiform

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encephalopathy.[5] Spongiform encephalopathies have also been described in a number of zoo animals including; kudu, nyala, Arabian oryx, Scimitar horned oryx, eland, gemsbok, bison, ankole, tiger, cheetah, ocelot and puma, which have developed coincident with the emergence of BSE.[2] Human prion diseases include Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker disease (GSS), Kuru[2] and fatal familial insomnia (FFI).[6] In 1995, a novel human prion disease, variant CJD (vCJD) was identified and subsequently found to be caused by the same prion strain that causes BSE in cattle.[7] This finding has fueled fears that dietary exposure to BSE prions may lead to a major epidemic of vCJD in the UK and other countries,[7] and has understandably stimulated considerable research interest in these diseases. A similar concern is presently raised in North America with the finding of BSE in Alberta and CWD in the western provinces and mid-western states.[8]

Scrapie was demonstrated to be a transmissible disease by inoculation experiments in sheep and goats in 1936.[2] Scrapie was initially believed to be caused by a "slow virus" infection, due to the prolonged incubation period.[2] Investigation into an epidemic of a neurodegenerative disease known as Kuru, affecting members of the Fore linguistic group in the Eastern Highlands of Papua New Guinea, incriminated cannibalistic feasts in the transmission of the disease.[9] Subsequently, Kuru, CJD and GSS were all successfully transmitted to chimpanzees, confirming the transmissible nature of these diseases.[2,10]

Classification of prion diseases

The prion diseases are classified etiologically, as acquired, inherited and sporadic. Acquired cases have resulted from cannibalism,[9] accidental exposure to infected tissues during medical or surgical procedures, such as via contaminated stereotactic electroencephalogram electrodes,[11] corneal transplants,[12] dura mater grafts,[13] and the use of human pituitary-derived gonadotrophin.[14] Dietary exposure to BSE has been responsible for an increasing number of vCJD cases, and this number may continue to increase as the incubation period is unknown.[7] This was quite surprising since epidemiological studies did not provide evidence for a link between scrapie and CJD in humans.[15]

Approximately 15% of human cases of prion disease are inherited and associated with mutations in the prion protein gene (PRNP).[16] Over 20 distinct mutations in the PRNP gene have thus far been identified.[17] These occur as missense mutations,[18,19] non-sense mutations,[20] or insertions in the octapeptide repeat region.[21] The main familial forms of prion diseases are classified as CJD,[22] GSS,[23] FFI[24] or familial atypical spongiform encephalopathy (FASE).[25] Despite all being associated with a single point mutation or insertional mutation in the prion protein gene, these diseases have unique clinical profiles and different parts of the brain are affected.[22]

The overwhelming majority of human prion cases, nearly 85%, have no clear evidence of a genetic mutation in PRNP nor documented exposure to an infectious agent and so are termed sporadic.[2] The majority of these cases are CJD,[26] although sporadic forms of GSS[27] and fatal insomnia[28] have been described.

Clinical Features and Pathology

Clinically Kuru and GSS cases present with ataxia, referable to the cerebellum, whereas, classical CJD presents as a rapidly progressive, multifocal dementia, usually with myoclonus.[2] FFI cases present with insomnia[24] and FASE cases suffer initially from memory problems.[25] Variant CJD is however, dominated by behavioral and psychiatric signs.[2] Scrapie in sheep is also marked in its early stages by behavioral abnormalities (i.e. withdrawal from normal flock societal behavior, nervousness, aggression) and pruritis, with progressive ataxia and trembling when excited.[29]

The classical triad of histopathological features of the prion diseases, spongiform vacuolation, neuronal loss and astrocytosis, are common to both animal and human diseases.[30] Amyloid plaques have also been described in some cases.[30] The incubation period, type, severity and distribution of these pathological changes, when scrapie strains are inoculated into a given strain of mice, are sufficiently specific to allow the identification of approximately 20 specific strains of scrapie.[31]

Vacuolation

Vacuolation is found within the perikarya and within neuronal processes.[32] It is a relatively common and prominent finding in both BSE and scrapie, though it is not invariably present.[33] In BSE, vacuolation is predominantly found in the paraventricular area of the hypothalamus, thalamus, periaqueductal gray matter, reticular formation, nucleus of the solitary tract, and the nucleus of the spinal tract of the trigeminal nerve.[34] In pigs infected with the BSE agent, vacuolation is most prominent rostral to the medulla, in the frontal cerebral cortex, striatum, thalamus, although it can also be found in the periaqueductal gray matter, tectum, with occasional vacuoles in the cerebellum.[35] The brainstem is also the area most commonly affected in sheep with scrapie, although the severity of the vacuolation varies with the breed of sheep.[36] The most consistent lesions are described in the reticular formation, dorsal nucleus of the vagus, lateral cuneate nucleus, and when the spinal cord is affected, in the dorsal horn and intermediate gray matter.[36] Vacuolar change is an infrequent finding in transmissible mink encephalopathy.[32] Vacuolation may also be inconspicuous in cases of classical CJD.[37]

In murine models of scrapie, vacuolation is not prominent early in the disease process but increases rapidly in the terminal stages of the disease.[38] The location of the vacuolation varies with scrapie strain in mouse models, however, the cerebellum is generally affected as a late event.[39] There is however, one strain of scrapie, 22L, that produces significant cerebellar lesions when injected into mice.[31] BSE when inoculated into mice, also produces cerebellar lesions.[40]

Vacuoles may also occur in oligodendrocyte cytoplasm,[38] within the myelin sheath[41] and in astrocytes.[42,43] The vacuoles may be single membrane bound,[44] double membrane bound,[41] unbound[42,43] or any combination of these in the same mouse model of the disease.[38] Baker et al[44] concluded that the single membrane bound vacuoles most likely originate in the smooth endoplasmic reticulum. Jeffrey et al[32] reported that vacuoles may also originate from mitochondria, or from within processes formed by the disassembly of microtubules and neurofilaments. Liberski et al[45] demonstrated that the intraocular injection of

tumor necrosis factor produced lesions in the mouse optic nerve similar to the changes seen in the myelin sheaths of panencephalopathic type of CJD. The appearance of the vacuoles is not closely linked with clinical disease as agent replication and tissue infectivity precede vacuolation[40,46,47] and nonvacuolated areas of the brain may still be infectious.[48] In hamster models of scrapie, clinical signs may precede the appearance of vacuolation.[47] Vacuolation is reduced in mink with the Chediak-Higashi gene[49] and in beige mice.[32] These mutations are associated with abnormalities in membrane bound organelles, including melanosomes and lysosomes, as well as a deficiency of lysosomal enzymes.[32] Vacuolation is also a feature of accelerated aging in mice[50] and has also been reported in experimental rabies infections in skunks and foxes,[51] as well as in retroviral infections in mice.[52] Taken together, these findings suggest that the vacuolation in transmissible spongiform encephalopathies (TSEs), one of the cardinal signs of the disease, is not the result of a pathologic change in a specific organelle but rather a more general feature of degeneration within the nervous system. The variable appearance of vacuolation and its lack of specific consistent temporal relationship with infectivity or clinical signs also suggest that it is a marker of degeneration rather than the specific cause of the pathological changes or clinical signs.

Neuronal loss

Neuronal loss is considered to be part of the classical triad of features of the TSEs, however, reliable documented evidence of this change is difficult to find. In many cases of CJD and GSS neuronal loss has not been objectively assessed.[53] Neuronal loss has not been unequivocally described in natural scrapie.[53] It has however, been described in BSE[54] and in murine scrapie.[55] Several features described in CJD may suggest neuronal injury including; irregular dendrites with reduced spines, dendrites with constrictions and dilations along their processes, axonal dystrophy, frequent formation of ubiquitin protein conjugates and neuronal autophagy.[53] Neurofilament proteins are often abnormally phosphorylated.[53] These findings suggest that neuronal loss is not the major cause of the clinical signs noted in the TSEs, but rather indicates that cell death is the result of a pathological process during which the neuron loses its ability to perform its function. Furthermore, this suggests that the pathological process in the disease is degeneration rather than cell death or apoptosis. In the early stages, degeneration causes dysfunction of the cell and is reversible. If the cells in question are neurons, profound symptoms or clinical signs may result, although the neurons may still be present. However, if the insult that initiated the degeneration continues, the cell will reach a point where it is unable to compensate and fulfill its physiological functions and cell death or apoptosis will result. The neuronal loss described in BSE, as well as in scrapie and BSE inoculated mice, may suggest a more virulent pathogen or more susceptible host. In these cases, the time for degeneration of the neurons prior to cell death is extremely short.

Astrocytosis

Although part of the "classical triad" of TSE pathologic findings, astrocytosis is a variable but sometimes prominent feature.[56] Mild astrocytosis is present in many natural cases of scrapie and in some GSS patients, though it can be severe in many cases of CJD.[57] Astrocytosis is present in most neurodegenerative diseases and is one of a limited repertoire of responses to injury in the central nervous system (CNS).[32] Ultrastructural and immunohistochemical features of astrocytosis include increased expression of glial fibrillary acidic protein, vimentin and the accumulation of glycogen, features that are typical of reactive cells.[58] Therefore, astrocytosis is most likely a response to the degenerative process induced by the TSE agent, rather than a specific response to the TSE agent itself.

Many reports indicate that there is no significant immune response with scrapie infection.[59] However, in cases with vacuolation or disease specific PrP accumulation, increased numbers of microglial cells have been reported[32] and confirmed in mouse scrapie models.[60] Scrapie infection in mice results in induction of IL-1β and TNFα in glial cells,[60] as well as NF-κB, the major transcription activator for inflammatory cytokines.[61] These findings do not rule out a more subtle immune response in the CNS, perhaps mediated by activated microglia and cytokines.

Amyloid plaques

Amyloid plaques were first described in kuru and were reported most consistently in the cerebellum.[62] However, they are only rarely found in scrapie and BSE, and when present, are usually present in perivascular locations.[34] Immunohisto-chemical studies have demonstrated that these plaques contain amyloid fibrils composed of PrP[63] and astrocytic and microglial processes.[64] The cell processes become a more prominent component of the plaques over time.[53] Ultrastructural and immunocytochemical studies have shown that PrP accumulates in astrocytic and microglial lysosomes, suggesting that these cells are involved in the phagocytosis and removal of abnormal or excess PrP, rather than in its production.[53]

Etiologic Agent

Protein-only theory

Numerous transmission studies have indicated that an infectious agent causes these diseases. The long incubation period between inoculation and the development of the disease led to the concept of a "slow virus" being the causative agent.[2] Alper et al[65] used the ability of ionizing radiation to inactivate biological activity, and determined that the agent may not contain nucleic acid and if it did, it must contain less than 800 bases. Subsequently, Pattison and Jones[66] suggested that the agent might be associated with a small basic protein. More recently, based on titration and transmission studies, the scrapie agent has demonstrated resistance to many treatments that degrade nucleic acids. It is resistant to wide changes in pH, nucleases, phosphodiesterases, ultraviolet irradiation at 254 nm, divalent cation hydrolysis with $Zn(NO_3)_2$, photochemical inactivation with psoralens and inactivation by hydroxylamine.[47] While there are viruses that are resistant to some of these procedures, there is no known virus resistant to them all.[47] However, these findings do not rule out the possibility of a virus-like agent. In contrast, infectivity of the agent is reduced following treatments that denature proteins, such as; digestion with proteinase K or trypsin, chemical modification with

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diethylpyrocarbonate, treatment with sodium dodecyl sulfate (SDS), chaotropic salts such as guanidinium thiocyanate, phenol, and urea.[47] Taken together, these observations led Stanley Prusiner to propose that the agent responsible for the TSEs was devoid of nucleic acids and was composed solely of protein, the "protein-only" hypothesis. This was clearly a unique concept. Prusiner[47] introduced the term "prion" to describe the agent. The purported prion protein was subsequently isolated and found to be a sialoglycoprotein, containing a 27 to 30 kDa core that was resistant to proteases, which became known as PrP[27-30].[67] Partial amino acid sequencing and cDNA construction led to the discovery that the prion protein was encoded by a single-copy chromosomal gene rather than the DNA of an infectious agent.[68] The prion protein gene (PRNP) encodes a 33-35 kDa protein (PrP[C]) that is sensitive to proteases.[69] The PrP[27-30] protease-resistant form is known as the scrapie protein (PrP[Sc]) and has the same amino acid sequence as the normal cellular form PrP[C], from which it is derived by posttranslational modification.[69,70] Comparison of PrP[C] and PrP[Sc] structure reveals that PrP[C] contains 42% α-helix and only 3% β-sheet, whereas PrP[Sc] contains 43% β-sheet and 30% α-helix.[71] The protein-only hypothesis proposes that PrP[Sc] acts as a template to change the conformation of PrP[C] into the PrP[Sc] form and PrP[Sc] is inextricably linked with the disease state.[17,72,73] The theory has been modified to suggest that another protein, "Protein X" may act as a facilitator in this conversion.[73,74] While the protein only theory has gained wide acceptance, the theory that the scrapie agent contains a virus-like nucleic acid is still championed by some researchers.

Resistance to UV and ionizing radiation does not prove that the scrapie agent does not contain nucleic acid, only that is must be small.[65,75] Narang[76] reviewing the resistance data, states that the data does not preclude the presence of a virus and since conventional viruses depend on their protein coat for their integrity and infectivity, decreased infectivity as a result of treatments that denature proteins does not prove that nucleic acid is not present. Moreover, Narang interprets the data as being supportive of the presence of nucleic acid in the agent, as DNA is known for its tremendous stability and biologically active DNA has been amplified from fossils and tissues that have been fixed in formalin and maintained in a paraffin block for over 40 years, conditions that proteins cannot withstand. Ultra-structurally, scrapie-associated fibrils and tubulofilamentous virus-like particles (nemavirus particles, NVP) are consistently seen in TSEs.[76] These are interpreted to be ultrastructural markers whereas PrP[Sc] is stated to be a protein marker of the agent.[76] Narang[76] argues that the protein-only hypothesis is based on negative and indirect evidence and further, that PrP post-translational modification is a pathological process and that PrP[Sc] is a by-product of the disease process rather than the agent itself. Narang[76] proposes that the agent contains a nucleic acid that encodes the "protein X", the protein now believed to be required by the Prusiner group to facilitate conversion of PrP[C] to PrP[Sc]. Single stranded DNA (ssDNA) of 1.2 kb has been purified from homogenized scrapie-infected hamster brain tissue using standard phenol/chloroform extraction and alkaline gel electrophoresis.[77] This ssDNA has a multi-palindromic structure that may explain its tremendous stability.[76] Narang claims that this ssDNA has been purified and injected into hamsters, and has

reproduced the disease.[76] He suggests that the ssDNA with the help of a carrier protein transmits the disease and that incorporation of the ssDNA into host DNA could explain the familial inheritance of scrapie in sheep, as well as inherited cases of CJD and GSS. However, these results have not been independently reproduced by other researchers.

Perhaps the most serious challenge to the protein-only theory has been raised as a result of the observation that the prion from a donor species, passaged in other species and then reisolated and injected into mice, retains its original amino acid sequence and phenotypic properties, and does not acquire the amino acid sequence of the PrP[C] from the intervening species.[78,79] Thus far, approximately 20 distinct scrapie strains have been identified via mouse inoculation tests.[31] As these strains can be identified by passage through mice of a single genotype, strain variation must be specified by the agent and be independent of the host.[31] These observations are difficult to reconcile with a single PrP[Sc]. The Prusiner group argues that the different strains can be explained by different posttranslational modifications of the PrP[C] and structures induced by the PrP[Sc].[17]

Toxin or metabolic disorder theory

Another interpretation of the data by Rico[80] yields an alternative theory regarding the agent. Rico argues that when considering PrP[Sc], one must also consider the way that water interacts with globular proteins, as water participates in the macromolecular structure of proteins. PrP[Sc] is strongly hydrophobic, water insoluble and surrounded by a micro-anhydrous environment, and probably a 1 nm thick gas cap, that is most likely composed of CO_2.[80] Rico argues that the absence of water inhibits interaction with proteases and chemical deactivators that function in an aqueous medium, and also explains the resistance to heat and radiation. Rico suggests that PrP[Sc] interacts with PrP[C] to disrupt the post-translational modification of the protein following its endocytosis and recycling from the membrane location. Structural rigidity and hydrophobicity may also explain the absence of an immunological reaction.[80] Based on these interpretations, Rico proposes that the inherited or sporadic spongiform encephalo-pathies should be considered as metabolic disorders and the acquired transmissible spongiform encephalopathies as intoxications. This is a conceptually different interpretation of the data that may also explain the inability to definitively detect nucleic acid in the agent.

Virino or virus theory and other infectious agent theories

The "virino" hypothesis suggests that the agent has an informational molecule, most likely ribonucleic acid (RNA), that is independent of the host, and that host derived PrP provides the protein component to protect the genetic material.[81] Heat inactivation of TSE infectivity exhibits biphasic properties, and is consistent with a theory whereby the agent contains two components.[79] In addition, Lasmézas et al[40] reported that 55% of mice inoculated with a homogenate of BSE-infected cattle brain, exhibited clinical signs and infectivity, even with three subsequent passages, however, there was no detectable abnormal form of PrP. These results suggest that infectivity and the abnormal prion protein can be dissociated and led Lasmézas et

al[40] to suggest that there may be an infectious agent in addition to PrP and that this agent may be a nucleic acid. Lasmézas et al[40] concluded that the abnormal prion accumulation was related to adaptation to a new host species, and that vacuolation and gliosis were linked to the abnormal protein accumulation. These transmission studies need to be replicated by other researchers in order to fully evaluate their significance. In addition, lack of sensitivity of the detection methods must be ruled out as a cause of the inability to detect abnormal prion protein in these transmission studies.

Manuelidis and Manuelidis[82] inoculated hamsters with buffy coat samples from healthy human volunteers with no family history of CJD and were able to produce spongiform changes in the brain of inoculated animals. Brain samples from similar healthy humans did not produce disease in hamsters. The authors interpreted these findings as suggestive of a viremia being present in most people within circulating leukocytes, but disease only being seen in a very small number of people.[82] Subsequent work using centrifugation in various solutions and treatment with guanidine hydrochloride or SDS on CJD infected brain samples led the authors to conclude that the CJD "agent" is a virus.[83] Another group has apparently isolated from TSE's an unconventional bacterium from the genus *Spiroplasma* that lacks a cell wall, and have proposed this agent as a candidate causative pathogen for these diseases.[84]

Clearly, the exact nature of the scrapie agent has not been universally accepted at this point, and so one must keep an open mind.

Pathogenesis

Sporadic and inherited forms of disease

The spongiform encephalopathies are thought to be the result of the accumulation of an abnormal isoform of the cellular prion protein, PrP^{Sc}.[85] In the inherited diseases, the abnormal isoform accumulates as a result of an autosomal dominant genetic mutation in the PRNP gene, presumably resulting in a protein with a propensity to misfold into the abnormal isoform, however, direct experimental evidence to support this theory is lacking.[86] The sporadic diseases account for the majority of the human cases of spongiform encephalopathy and are believed to be caused by a rare biochemical event that initiates the misfolding of the PrP^C into the pathologic isoform.[87] Proposed initiating metabolic events include misrouting to the cytoplasm,[88] deamidation and[89] interaction with bivalent manganese ions.[90]

Acquired forms of disease

Regardless of the nature of the etiologic agent of TSEs, it is transmissible between hosts. Understanding the mechanism of transmission is the first step towards understanding the pathogenesis of the disease. In cases of accidental transmission via medical or surgical procedures involving contaminated tissue, or in cases of experimental injection into the cerebrospinal fluid, direct transmission from nervous tissue to nervous tissue is obvious. However, in TSEs believed to be transmitted via the oral route, such as BSE, vCJD and transmissible mink encephalopathy, the route of neuroinvasion is less clear.[91] Recent evidence suggests that after absorption from the gastrointestinal tract, the agent is carried via the systemic circulation to the spleen, where it invades mature follicular dendritic cells (FDC) of the white pulp.[92] The FDCs are a prominent site of PrP^{SC} deposition.[93] Maturation of the FDCs is directed by cytokines released by B lymphocytes and so both cell types are critical for the propagation of the agent.[92] In fact, depletion of FDCs prevents neuroinvasion[94] and various B lymphocyte defects inhibit neuroinvasion.[91] From the FDCs, the agent is believed to enter sympathetic nerves and from there, disseminate to the CNS.[93] Consistent with these observations, hyperinnervation of the spleen results in significantly greater replication and neuroinvasion, whereas, denervation of the spleen delays or prevents neuroinvasion.[93] Beekes et al[95] have suggested that following absorption from the GI tract, neuroinvasion via the vagal nerve to the dorsal motor nucleus is another possibility.

According to the most widely accepted protein-only hypothesis of Prusiner, PrP^C acts as substrate for PrP^{Sc}-mediated conversion of PrP^C into PrP^{Sc}, with "Protein X" acting as a catalyst in the process. The requirement for a species specific protein required to facilitate the conversion of PrP^C to PrP^{Sc} was deemed necessary on the basis of studies with mice expressing either mouse PrP^C alone, mouse PrP^C and human PrP^C and mice expressing a chimeric mouse/human PrP with or without expression of mouse PrP^C.[74] The catalyst protein termed "Protein X" provided the best explanation for the transmission studies in these mice.[74] Two models have been proposed to explain how PrP^C may be converted to PrP^{Sc}. The first is the template-directed model, whereby a PrP^{Sc} monomer would initiate the conversion, perhaps aided by protein X. In this model, PrP^{Sc} would be more stable than PrP^C, but kinetically inaccessible.[96] The second model is the nucleation-seeding model. In this model, a nucleus of aggregated PrP^{Sc} initiates polymerization and conversion of PrP^C, perhaps aided by protein X. In this model, monomeric PrP^{Sc} would be less stable than PrP^C, but would be stabilized upon joining the PrP^{Sc} aggregate.[97]

PrP^C has been converted into a protease-resistant form by denaturation and incubation with PrP^{Sc}, however the product has not been demonstrated to be infectious.[98] More recently, PrP^C has been converted to a protease resistant form (PrP^{res}) *in vitro* using the incubation of PrP^{res} with normal brain homogenate and then subjecting the mix to repeated cycles of sonication via a protein misfolding cyclic amplification assay (PMCA).[99] The *in vitro* generated product has demonstrated infectivity when inoculated into wild-type hamsters.[99] Curiously however, the "purified" product is less infectious than the equivalent dilution of infectious crude brain extract.[99] These experiments, although showing that PrP^C can be converted to PrP^{res} were done with the continued addition of normal brain homogenate and therefore do not rule out the presence of another factor in the brain homogenate that may be required for disease. In the protein-only theory, PrP^C is required to provide a substrate to form the pathologic isoform PrP^{SC}, as has been demonstrated in the Prnp knockout studies.[100] Reducing the amount of available PrP^C via antibodies or inhibitors therefore, seems a logical therapeutic rationale, however, before such an approach should be undertaken, the physiological functions of PrP^C must be thoroughly investigated.

In order to investigate the neurotoxicity of PrP^{Sc}, Brandner et al[101,102] grafted brain tissue expressing PrP^C into the brains of

Prnp[0/0] mice, which do not express PrP[C], then inoculated scrapie prions intracerebrally. The grafts developed severe neurodegenerative lesions as would be expected in a wild-type mouse, however, despite high levels of PrP[Sc] in the grafted tissue and throughout the brain, no pathological changes were found in the adjacent brain not expressing PrP[C]. The PrP[Sc] isolated from these brains was demonstrated to be infectious. Brandner et al. concluded that PrP[Sc], by itself, is not neurotoxic. Interestingly, when they used an intraperitoneal route of infection, they were not able to demonstrate prions in the spleen and there was no sign of disease in the grafted brain tissue.[103] They concluded that a PrP[C] expressing tissue is required to facilitate the invasion of prions into the brain. Irradiation of the bone marrow of these Prnp[0/0] mice, followed by bone marrow grafts from wild-type mice, was able to elicit infectivity in the spleen following intraperitoneal challenge, but no disease or infectivity was found in the PrP[0/0] brain. They concluded that transfer from the spleen to the CNS requires another tissue, most likely the peripheral nervous system. Other experiments in severe combined immunodeficiency mice, reconstituted with wild-type spleen grafts restored susceptibility to scrapie after peripheral inoculation.[104] Other experiments with various models of immunodeficiency demonstrated that differentiated B lymphocytes play a crucial role in neuroinvasion, whereas, there was no requirement for T lymphocytes.[91] The same group demonstrated that the role of B cells seemed to be related to their role in inducing maturation of the follicular dendritic cells (FDCs), as prion immunoreactivity was localized to the FDCs and not the B cells.[105] Overall, these findings suggest that components of the immune system are required for neuroinvasion of scrapie and that the FDCs are the most likely prion reservoir in the immune system.

In experimental scrapie infections of mice the first demonstrable indication of the presence of the agent is the extracellular accumulation of disease-specific PrP[Sc] in the hippocampus at around day 70 post-infection.[106] Ultrastructural evidence of synaptic loss is present around day 84 post-infection, with axonal terminal degeneration and vacuolation occurring around day 98 post-infection.[106] The synaptic disruption was also associated with the loss of dendritic spines and abnormal dendritic morphology.[107] Siso et al[108] found an overall decrease of proteins associated with synaptic function, such as synaptophysin, SNAP-25, syntaxin-1, α-synuclein, and β-synuclein in scrapie-infected mice. Taken together, these data provide evidence for both pre and post-synaptic pathological changes associated with the disease, which would disrupt neuronal circuitry, perhaps leading to neuronal degeneration and eventually, apoptosis.

Biological Features of the normal cellular Prion protein (PrP[C])

PrP[C] gene

Isolation of the 27 to 30 kDa protease resistant prion protein (PrP[sc]), and its partial amino acid sequencing, followed by cDNA construction, led to the discovery that the Hamster prion protein was encoded by a single-copy chromosomal gene rather than an infectious agent.[68] Subsequently, Kretzschmar et al[109] cloned the human PRNP gene, which is located on the short arm of chromosome 20.[110] The human gene locus contains a 134 bp exon I and a 2355 bp exon II, separated by a 12,696 bp intron.[111] The entire coding region is located in the second exon.[109] The PRNP promoter region is typical for a housekeeping gene, with a C_pG island extending from –235 to +167 bp within intron I and lacks a canonical TATA box and an initiator element.[112] Based on the sequence of the promoter, binding sites for transcription factors such as nuclear-factor interleukin 6 (NF-IL6), muscle-specific factor MyoD, S_p1, heat shock factor, AP-1, AP-2 are present, and many are well conserved among mammalian species. The presence of the NF-IL6 binding site may suggest that proinflammatory cytokines may play a role in PrP[C] expression. Using functional analysis of the promoter region, deletion of the region from –148 to –114 resulted in a 62% drop in gene expression in human neuronal (embryonic retinal) cells but not in HeLa (human uterine carcinoma) cells, suggesting that this region may be particularly important in neuronal cells.

Familial mutations and polymorphism influence disease

A number of amino acid mutations in PrP[C] in humans appear to influence the onset and phenotype of human inherited prion diseases.[17] Over 20 amino acid mutations are reported, most of which are proposed to destabilize the PrP[C] structure.[17] However, studies on the thermodynamic stability of some of these mutations do not support this hypothesis.[86,113] Prion proteins with insertional mutations (3 or 5 additional octapeptide repeats) have a more exposed N terminal and are more susceptible to oxidative damage.[114] Apetri et al[115] found that increased thermodynamic stability of an intermediate form in the folding process of the prion protein (relative to the wild-type protein) is present in 7 out of 9 familial mutations examined and suspect that this may be a crucial factor in the conversion of these proteins to a disease associated form. However, the intermediate form for 2 out of the 9 (E200K and P102L) did not show this increased stability, indicating that this property is not consistent and so either its relationship to the disease state is coincidental rather than causative or there exists several mechanisms that lead to prion diseases associated with different familial prion protein mutations.

A polymorphism at position 129 has also been reported in humans, where approximately 40% of Caucasians are homozygous for methionine, 10% homozygous for valine and 50% heterozygous.[116] Homozygosity at position 129 appears to predispose to sporadic CJD.[117] Interestingly, all clinical cases of variant Creutzfeldt-Jakob Disease (vCJD) thus far identified, are homozygous for Met 129.[118] A single preclinical case in a heterozygous Met/Val at codon 129 patient that died from a ruptured abdominal aortic aneurysm five years after receiving a blood transfusion from a patient that had subsequently developed vCJD has been identified.[119] This patient had PrP[res] present in the spleen, but not in the central nervous system, nor was there evidence of pathological features associated with vCJD. An aspartate substitution for an asparagine at position 178 is also associated with either Fatal Familial Insomnia (FFI) or familial Creutzfeldt-Jakob Disease (fCJD), depending on whether there is a methionine or a valine at position 129, respectively.[19] Heterozygosity at codon 129 appears to delay the onset of inherited prion diseases and these data suggest that it may also be protective against vCJD.[44,116]

Predictably, other structural changes are also associated with diseases. For example, a higher number of octapeptide repeats is associated with earlier onset of disease[7] characterized by dementia and ataxia.[120]

PrP protein

Protein expression

The PrP^C protein is expressed in a wide variety of tissues including the brain, spleen, lymph nodes, kidney, pancreas, salivary gland, adrenal gland, liver, thymus.[90,121] testes, lung[68] and muscle.[122] It is present in at least 27 mammalian species, birds[123] and reptiles.[124] A unique form of PrP^C, truncated at the C-terminus is expressed only in rodent, cattle and human spermatozoa.[125] The expression of PrP^C increases with age in mice.[126] Human PrP^C is a 253 amino acid precursor protein encoded from an intronless open reading frame[127] and has a molecular weight of 25 to 35 kDa.[128] The protein contains 254 amino acids in the mouse.[111]

Not only is PrP^C ubiquitously expressed, but key elements of the protein structure are also highly conserved. The human protein has four identical proline and glycine-rich tandemly repeated octapeptides (ORs) at the N-terminus,[120] a central transmembrane domain and a stop transfer effector domain (STE).[70,128] However, the number of ORs in homozygous individuals varies among species from four in humans[120] and golden mole, up to seven in the gymnure and leaf-nosed bat.[127] Many motifs involved in post-translational modification of the protein (see below) are also conserved among species; glycosylation sites, the hydrophobic transmembrane domain, structural elements for N and C-terminal processing and attachment of the GPI anchor to the outer cell membrane.[127]

Post-translational modification

PrP^C is directed cotranslationally into the lumen of the endoplasmic reticulum (ER), a process that is mediated by a 22 amino acid N-terminal signal peptide (Figure 1).[129] Within the ER, the N-terminal signal sequence is removed, as is a 23 amino acid C-terminal sequence, which facilitates the addition of the GPI anchor for attachment to the cell membrane. A disulfide bond between Cys 178 and Cys 213 is essential for proper folding of the protein.[130]

A particularly important post-translational modification involves N-glycosylation, which has been mapped to Asn 180 and Asn 196 in the human protein.[131] High-mannose glycosylation and addition of the GPI anchor occur concurrent with or soon after translation and translocation of polypeptides into the lumen of the ER.[132] The N-linked oligosaccharide chains that are added in the ER are rich in mannose and as such, are sensitive to endoglycosidase H digestion.[133] These oligo-saccharides are later modified by the addition of sialic acid in the Golgi and are resistant to digestion with endogycosidase H. Final maturation of glycosylation requires transit to the Golgi apparatus where N-acetylneuraminic acid is added to the GPI anchor.[134] Caughey et al[132] described three main glycoforms of the protein, fully glycosylated, monoglycosylated and ungly-cosylated, which can readily be identified by western blotting of protein extracts. However, given that glycosylation occurs in the ER as PrP is being translated, and transit to the Golgi is required

to add N-acetylneuraminic acid, these three main glycoforms could also represent mature glycosylated and immature glycosylated forms. It would therefore be advisable to check the status of PrP glycosylation with endoglycosidase H or N-glycosidase F (PNGase F) in order to clearly understand glycosylation of PrP in various situations. Glycosylation is thought to play an important role in intracellular trafficking of the protein,[135] and may also play a role in the folding or misfolding of the protein.[136] Indeed, N-glycosylation is believed to help stabilize the normal structure of PrP^C.[137]

Three topological forms of PrP^C have been identified.[128] Using cell-free translation systems containing ER-derived microsomal membranes, Hegde et al[128] identified a C-transmembrane form (^{Ctm}PrP) where the C-terminal is within the ER lumen and following the normal maturation and transit of the protein through the Golgi apparatus, would be expressed on the extracellular surface, an N-transmembrane form (^{Ntm}PrP), where the N-terminal is within the ER lumen and would be expressed on the extracellular surface, and a secreted form, entirely within the ER lumen, where it would be attached to the cell surface via the GPI anchor. The authors suggested that ^{Ctm}PrP might be associated with disease. Hegde et al[128] also described the secreted PrP (PrP^C) and ^{Ctm}PrP forms as being glycosylated and the ^{Ntm}PrP form as being unglycosylated.

Other post-translationally modified forms of PrP have also been described. Stahl et al[134] identified six different glycoforms via a two-dimensional immunoblot technique. Using a similar technique, Pan et al[138] identified seven PrP^C forms based on molecular weight alone. Each of these generated from 3 to 14 distinct spots on a gel as a result of different charges on the molecule.[138] These different electrical charges could be due to different glycans, slight differences in the primary structure due to truncations or due to differences in the composition of the GPI anchor.
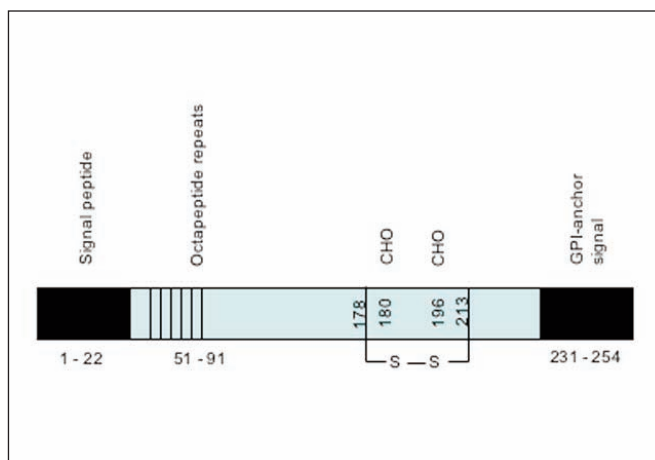

Figure 1: *Schematic diagram of PrP*

Clearly, there is a potential for a bewildering array of PrP forms arising from a single primary structure, due to post-translational modification of the protein. Glycosylation in particular is thought to play an important role in the normal trafficking of PrP and perhaps in the relationship of PrP[C] to disease.
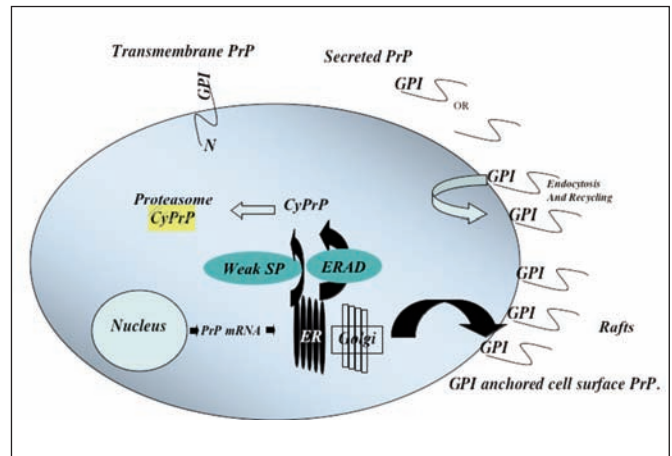
Normal trafficking of PrP

Following translation, glycosylation and the addition of the GPI anchor, in neuroblastoma lines, approximately 90% of the protein is transported to the cell surface where it is attached via the GPI anchor[70] to the outer cell membrane (Figure 2).[127] Also in neuroblastoma cells, the half-life on the cell surface is approximately three to six hours.[132] The PrP[C] molecules are transported to the cell surface in association with cholesterol rafts[139] and indeed, cholesterol is required for cell surface localization.[140] Rather than being simply a transport vesicle however, lipid rafts may have a role in the folding of PrP[C].[141]

From the cell surface, some of the PrP[C] is internalized into an endosome, from where a C-terminal fragment may be recycled to the surface,[142] however, Shyng et al[143] reported that most of the protein is recycled without degradation. This endocytosis may be initiated by copper binding via the octapeptide repeats[144,145] or not require copper binding.[146] Internalization occurs via clathrin-coated pits[147] and/or caveolae-like membranous domains[148] or sphingolipid/cholesterol rafts.[146] The N-terminal may play a role in modulating endocytosis.[129,146] Clathrin-mediated endocytosis involves the recruitment of clathrin and adaptor proteins, such as AP-2 at phosphoinositides in the membrane.[149] However, impairment of clathrin-mediated endocytosis with protein mutants did not affect the internalization of the GPI anchored PrP suggesting that this mechanism may not be the major mechanism for all GPI anchored proteins.[150] Shyng et al[151] used hypertonic media to disrupt clathrin lattices and thereby impair endocytosis via clathrin and reported impaired PrP[C] internalization, suggesting that PrP[C] may not behave like other GPI anchored proteins. Internalization of proteins through caveolae has been suggested to divert proteins from the endosomal/lysosomal pathway.[152] Nichols et al[150] showed that GPI anchored proteins may use caveolae to traffic from the cell membrane to the Golgi. PrP[C] has been reported in endosomes containing transferrin receptors in adult mouse sensory neurons and N2a neuroblastoma cells.[146] Also in neurons, PrP[C] has been demonstrated both in the Golgi and within cytoplasmic organelles resembling endosomes.[153]

Although the majority of PrP[C] is expressed on the cell surface,[70,154] significant amounts are present within the cytoplasm of a subpopulation of neurons in the cortex, hippocampus and thalamus.[154] While some of this cytoplasmic PrP may arise from endocytosed cell surface PrP, some may also represent cytosolic PrP derived from the endoplasmic reticulum associated degradative (ERAD) pathway. Indeed, several investigators have reported the accumulation of PrP in the cytosol of cells treated with proteasomal inhibitors[88,155-158] indicating that misfolded or excess PrP[C] may be delivered to the cytoplasm and degraded by the proteasome system.[157,159] However, because of a weak signal peptide, PrP can also be translated as a cytosolic protein after losing its signal peptide.[160] Moreover, overexpression of PrP in cells often bypasses the secretory pathway to generate cytosolic PrP containing both the N-terminal and C-terminal signal peptides.[161] Ma and Lindquist further reported that cytosolic PrP becomes resistant to proteinase K and is toxic to N2a neuroblastoma cells and cerebellar granule neurons and proposed that cytosolic PrP may act as a seed for prion diseases.[156,162] The proportion of PrP[C] that is normally present in the endolysosomal compartment[163] or in the cytosol has thus far not been determined.

Taken together, these data indicate that there are several pathways by which PrP[C] can be recycled from the cell surface and several subcellular compartments where PrP[C] could be present and could conceivably interact with the disease associated form of the protein. The role of copper in the process of internalization of PrP[C] has also not been definitively confirmed. The seemingly conflicting results from various studies may represent differences among neoplastic cell lines, primary cell cultures, in vivo systems, as well as experimental methods to block components of the system being studied.

Figure 2: Schematic diagram of PrP trafficking. PrP is normally synthesized through the secretory pathway and acquires proper folding, N-linked glycosylation and a GPI-anchor in the endoplasmic reticulum. Most of this protein accumulates at the cell surface.[250] Some of this cell surface protein undergoes endocytosis and can be recycled to the cell surface.[142] However, mis-folded PrP is also known to undergo retro-translocation into the cytosol, where it is degraded by the proteasome.[155,157,162,251] In addition, the weak signal peptide of PrP can result in cytosolic PrP for proteasome degradation.[160] In addition to the cytosolic and cell surface GPI anchored PrP, some PrP is released in the extracellular milieu and a small fraction remains as a transmembrane form although the subcellular location of this form is not known.[248,252,253]

Proposed Physiological Functions of PrP

The physiologic role of PrP[C] has been the subject of considerable debate. PrP[C] is attached to the outer cell surface and found throughout the cell surface[153] and specifically at synaptic areas in neurons,[164,165] which may suggest both a generalized

role in cell metabolism and perhaps a specific role in synaptic transmission. PrP[C] is also found within the cytoplasm.[154] Therefore, physiological roles for PrP[C] within the cytoplasm, at the cell membrane and at synapses in neurons all seem possible. The physiological roles of PrP[C] must be thoroughly investigated before reducing PrP[C] availability should be considered as a potential therapy in the prion diseases. The generation of the subtle phenotype reported in Prnp homozygous knockout mice, does not definitively rule out a role for the loss of function of PrP[C] in the development of the disease, as a phenotype generated by knockout procedures only demonstrates those deficits that cannot be compensated for by other systems. The post-natal knockout using the Cre-*lox*P system, knocking out PrP in neurons after nine weeks, is more convincing,[166] although the loss of PrP[C] function simply via the loss of gene expression in mice does not test exactly the loss of PrP[C] function under specific stress conditions.

Copper metabolism and anti-oxidant role

Divalent copper binds to PrP[C] via the octapeptide repeats present in the N-terminal region.[167] Two of these binding sites may be physiologically relevant with Kd values around 10^{-14} and 4×10^{-14} M, which are comparable to other copper binding proteins such as superoxide dismutase and ceruloplasmin.[168] However, van Rheede et al[127] reported that not only do the number of octapeptide repeats vary among species, but also the number of histidine residues, which are implicated in Cu^{2+} binding vary, which suggests that copper binding may not be the primary function of the PrP[C] protein.

Copper binding to PrP[C] is thought to stimulate its internalization,[144] perhaps via a clathrin-mediated mechanism,[147] and has been proposed as the determining factor in PrP[C] internalization.[169] Copper binding appears to be pH dependent, which combined with the internalization following binding, suggests a role in Cu^{2+} absorption.[168] PrP[C] has been proposed as a modulator of the activity of Copper (Cu^{2+})/Zinc (Zn^{2+}) superoxide dismutase, a key intracellular antioxidant enzyme.[170] Moreover, levels of PrP[C] seem to correlate with Cu/Zn superoxide dismutase activity[170,171] and glutathione reductase activity.[171] However, other researchers do not find that PrP[C] has dismutase activity and conclude that if it has a role in the protection against oxidative stress, it is an indirect one.[172]

Neuronal cell cultures from Prnp[0/0] mice are more susceptible to oxidative stress than are wild-type (PrP^{+/+}) cells.[173,174] The PrP[0/0] neurons had significantly lower glutathione reductase activity that appeared to be modulated by PrP[C] expression.[174] Indeed Pereira et al[175] speculated that impaired brain anti-oxidant defenses may be a significant factor in the lower threshold for seizure activity noted in Prnp[0/0] mice. In addition, spermatozoa from Prnp[0/0] mice are more susceptible to copper toxicity than are spermatozoa expressing PrP[C]. This suggests a protective role for copper binding by PrP[C].[125] PrP[0/0] cell lines are also more sensitive to copper toxicity.[165] Brown[165] suggested that PrP[C] deficient cells may be able to compensate for the lack of the protein by using other copper uptake proteins, such as copper transporting receptor proteins.

Brown,[165]noting the synaptic concentration of PrP[C] in neurons and the copper binding affinity, proposed that PrP[C] has a protective role at the synapse rather than a direct role in neurotransmission. A possible anti-oxidant protective role at the synapse may be particularly significant in light of the fact that the earliest lesions in the prion diseases have been noted at the synapse.[106-108]

Overall, the data suggest that PrP[C] expression may contribute to Cu^{2+} binding and/or uptake, increased Cu/Zn superoxide dismutase and glutathione reductase activity and increased resistance to oxidant stress, moreover, the loss of function of PrP[C] may contribute to the pathogenesis of the prion diseases. However, given that a role for PrP[C] in the protection against oxidative stress is not uniformly accepted,[172] PrP[C] may not be concentrated at the synapse[154] and even that PrP[C] may be involved in Zn transport and homeostasis rather than Cu,[176] a significant role for PrP[C] in copper metabolism and as an anti-oxidant remains to be proved.

Signal transduction and growth

PrP[C] binds to a large number of proteins including, heat shock protein (HSP)60,[177] heparin-like compounds,[178] neuronal phosphoprotein synapsin Ib, a still uncharacterized prion interactor I (PintI),[179] as well as the carboxy-terminal decapeptide in the γ-1 chain of laminin, the most highly conserved of all laminin types.[180] Indeed, the 37 kDa laminin receptor protein has been proposed to act as a receptor for PrP[C].[181] This interaction is important for both extension and maintenance of neurites in PC12 and hippocampal neurons in culture.[180] Furthermore, cultured neurons from Prnp[0/0] mice extend fewer neurites than wild-type cultures when grown on media containing laminin, which are unaffected when exposed to anti-PrP[C] antibodies, a procedure that inhibits neurite growth in wild-type cultures, suggesting that those neurons that do form do not express PrP[C].

A fusion protein of mouse PrP[C] and the F_C region of human IgG interacting with cerebellar granule neurons derived from C57BL/6 mice expressing the GPI-anchored form of mouse PrP[C], stimulated increased neuronal survival and neurite outgrowth compared to controls.[182] Moreover, Chen et al[182] also found that different members of the Src kinase family were involved in prion-mediated neurite outgrowth and neuron survival. More recently, Steele et al[183] found that neuronal differentiation is delayed *in vitro* and *in vivo* if PrP[C] is absent and suggest that PrP[C] may be a positive regulator of neuronal precursor proliferation.

PrP[C] is also reported to play a role in known cell-signaling pathways. PrP[C] binds to the adaptor protein (growth factor receptor binding protein) Grb2, which is involved in activating ERK1/2 and MAP kinases.[179] In a murine 1C11 neuronal differentiation model, PrP[C] activates the Src family member tyrosine kinase Fyn via a caveolin-1-dependent mechanism, suggesting a role in signal transduction.[184] PrP[C] activation of the cAMP/PKA pathway is neuroprotective.[185] The outcome of ERK-mediated or Fyn-mediated signaling is currently unknown. Zanata et al[186] speculated that STI1 may be the protein that binds to PrP[C] and facilitates its conversion to the disease-associated form and may therefore be the "protein X" of Prusiner's protein-only theory.[74]

Overall, these data indicate that PrP[C] is capable of binding to a variety of ligands and may potentially be involved in regulating a variety of signal transduction pathways, which affect cell

growth and differentiation. Conclusive evidence for such a role for PrP^C has not thus far been elucidated, but this appears to be a very active area of research. The expression of PrP^C in a wide variety of tissues may indicate that PrP^C is performing a similar role in other tissues by transmitting signals from the extracellular compartment to the intracellular milieu.

Homeostatic functions

Calcium metabolism and regulating neuronal activity

Observations made on PrP$^{0/0}$ mice suggest that PrP^C may be involved in regulating circadian rhythms and sleep patterns,[187] as well as memory retention in aging rodents.[188] PrP$^{0/0}$ mice are also known to have a lower threshold for seizure activity than their wild-type counterparts, which is believed to be related to an increased susceptibility to oxidative stress.[175,189] Recently, it has been proposed that PrP may play a role in the formation of long-term memory in humans.[190]

Evaluation of these observations at the electrophysiological level has implicated the regulation of calcium in their pathogenesis. In hippocampal slices from PrP$^{0/0}$ mice, Ca^{2+} activated K$^+$ currents are disrupted.[191] These channels are involved in late after hyperpolarizations and therefore may be important in long-term potentiation and memory consolidation, and may also have a role in the lower threshold for seizures reported in PrP$^{0/0}$ mice. Similarly, Herms et al[192] examined cerebellar granule cells and found alterations in both the basal Ca^{2+} concentration and the changes in Ca^{2+} concentration with K$^+$ depolarization in tissue slices from PrP$^{0/0}$ mice. Herms et al[193] examined Purkinje cells in cerebellar slices and found a correlation between PrP^C expression level and the amplitude of maximum calcium concentration after depolarization. It has therefore been proposed that PrP may play a role in regulating synaptic activity.[166,194]

However, another group has disputed this proposed role as they have failed to find the same electrophysiological abnormalities.[195] In addition, PrP^C does not appear to be concentrated at the synapse, as was previously thought.[154] Moreover, most of the electrophysiological abnormalities have been reported in the hippocampus, which is not involved in the "retention" of memory. Other neurobehavioral studies in mice have suggested subtle differences between PrP$^{0/0}$ and PrP$^{+/+}$ mice,[196] however, no attempt was made to interpret the findings and identify the location of any possible lesion. Given the inconsistency or purely descriptive neurobehavioral studies, the significance of these findings must be questioned. Overall these data suggest that PrP^C may have a role in regulating neuronal activity, perhaps via Ca^{2+} gated K$^+$ channels, but this proposal requires further study.

Also related to Ca^{2+} metabolism, Kristensson et al[197] infected mouse neuroblastoma cells with PrP^{Sc} and noted a disruption of mitogen-activated increases in intracellular calcium concentration. The authors interpreted these findings to suggest that PrP^C may be involved in regulating intracellular Ca^{2+} concentration. However, following PrP^{Sc} infection, neuroblastoma cells may undergo the process of degeneration and degenerating cells would not be expected to be "normal" with respect to their physiological processes. In other words, these findings could be the effect of the degeneration rather than the cause.

Nucleic acid binding

PrP also binds to nucleic acids and induces the production of large nucleoprotein complexes when mixed with viral nucleic acids *in vitro*.[198] Human or ovine PrP^C is able to functionally replace HIV-1 nucleocapsid protein NCP7 nucleic acid chaperoning during the retroviral life cycle. NCP7 is required for the initiation of reverse transcription.[199] An anti-retroviral role for PrP^C has therefore been proposed.[200]

However, PrP^C binding to nucleic acids may also have a negative side. Human recombinant PrP^C binds to synthetic small highly structured RNAs under *in vitro* though, physiological conditions.[201] The PrP^C then becomes resistant to digestion by proteinase K and the RNA in the nucleoprotein complexes becomes resistant to digestion by ribonuclease A. Moreover, Deleault et al[202] found that the addition of mammalian RNA species to normal brain homogenate seeded with prion-infected brain homogenate amplified the conversion of PrP^C to PrP^{res}, a form of PrP that may be associated with disease. This amplification was inhibited by the addition of RNase in a dose-dependent manner.

Taken together these findings may suggest that PrP^C could play a protective role against retroviral replication in the cell, but could also be a key element in the amplification of the disease-associated form of PrP. Clearly, considerably more research will be required to confirm the validity of these experimental results.

Involvement in the immune response

In addition to being abundantly expressed in the brain, PrP^C is also expressed in cells of the immune system.[121] Treatment with the T lymphocyte mitogen concanavalin A induced significantly greater proliferation in T cells from PrP$^{+/+}$ mice compared to PrP$^{0/0}$ mice.[203] Concanavalin A treatment also resulted in greater production of interferon (IFN)-γ and interleukin (IL)-2 in splenocytes from PrP$^{+/+}$ mice or splenocytes from PrP$^{0/0}$ mice that had been transfected with a PrP expressing plasmid than splenocytes from PrP$^{0/0}$ mice.[204] These two cytokines are critical in the type 1 immune response. Treatment with anti-PrP^C suppressed mitogen-induced lymphocyte activation.[205]

The culture of CD14$^+$ monocytes in the presence of IFN-γ resulted in increased expression of PrP^C,[206] suggesting that PrP^C may be related to increased activation of monocyte/macrophage cells. In contrast however, de Almeida et al[207] found that macrophages not expressing PrP^C were more efficient at phagocytosis using an in vivo peritonitis model than PrP^C expressing macrophages and concluded that PrP^C is a negative regulator of phagocytosis.

Expression of PrP^C was down-regulated upon differentiation of hematopoietic cells along the granulocyte lineage.[208] Zhang et al[209] also examined PrP^C expression in hematopoietic cells and found that PrP^C expression in stem cells is important in their self-renewal following serial transplantation, however, they found no differences in progenitor cell or terminally differentiated hematopoietic cell numbers in PrP$^{+/+}$ or PrP$^{0/0}$ mice *in vivo*. These findings suggest that the lack of PrP^C expression is overcome in PrP$^{0/0}$ mice, or perhaps defects may only be seen in aged mice. Alternatively, PrP^C expression may be important in the experimental paradigm but less so *in vivo*.

Taken together, these data suggest that PrP[C] may play an important role in the regulation of the type 1 adaptive immune response, a response that is obviously critical to survival of the organism. However, evidence for a role in the innate immune response or in hematopoietic differentiation and proliferation is inconsistent and no firm conclusions can be made at this time.

Cell survival

It is generally accepted that the disease-associated form of the prion protein has either a direct or an indirect role in the neurodegeneration culminating in neuronal death that is a feature of the transmissible spongiform encephalopathies. It is therefore, somewhat ironic that the normal form of the protein PrP[C] may be associated with neuronal survival, however there is increasing evidence that this may be the case.

Using a model for traumatic brain injury, Hoshino et al[210] reported that PrP[0/0] mice had a significantly larger lesion volume than did PrP^{+/+} mice and also that the breakdown in the blood brain barrier in PrP[0/0] mice was more extensive one month after the event. Similarly, in rodent models of cerebral ischemia, the ischemic penumbra was larger in PrP[0/0] mice than PrP^{+/+} mice.[211] In the early phase following either ischemic injury[211,212] or traumatic brain injury[213] PrP[C] was markedly upregulated. In addition, Spudich et al[214] reported larger infarcts in PrP[0/0] mice than in PrP^{+/+} controls and also found increased activities of ERK1/2, STAT-1 and caspase 3 in the PrP[0/0] ischemic brains, suggesting a possible signaling mechanism through PrP[C] that may provide neuroprotection. In an *in vivo* model involving the injection of anti-PrP antibodies into the hippocampus, massive neuronal apoptosis was noted 24 hours after treatment.[215] This may suggest that PrP[C] is involved in transmitting survival or anti-apoptotic signals into the neuron.

These findings suggest that PrP[C] may provide a neuroprotective role *in vivo* and may be particularly important in the early period after the ischemic or traumatic event. The promoter region of PrP contains binding sites for heat shock transcription factors,[112,216] which could therefore provide another possible mechanism for upregulation due to cell stress and subsequent neuroprotection.

Neuroprotection could also be related to protection against oxidative stress or related to apoptosis via an anti-Bax function. Kuwahara et al[217] found that PrP^{+/+} hippocampal cell lines survive serum-deprivation induced apoptosis better than PrP^{-/-} cell lines. Similarly, neuronal cultures derived from PrP[0/0] mice were more susceptible to apoptosis induced by serum deprivation than PrP^{+/+} neuronal cultures, an effect that was abrogated by the reintroduction of PrP to the PrP[0/0] neurons.[218] Moreover, Kurschner and Morgan[219] demonstrated that PrP[C] interacts with the C-terminal 37 amino acids of Bcl-2 by using a yeast two-hybrid system. However, the interaction between Bcl-2 and PrP could not be confirmed in a mammalian system. They suggested that Bcl-2 may be the "protein X" presumed to be involved in converting PrP[C] to PrP[Sc] in Prusiner's protein-only theory.[74]

Consistent with the anti-apoptotic function of PrP in mouse neuronal cells, Bounhar et al[220] found that PrP[C] protects human neuronal cells in culture from Bax-mediated cell death. Furthermore, the cytosolic form of PrP[C] prevents Bax-mediated death in human primary neurons.[155] Specifically, PrP inhibits the proapoptotic conformational change of Bax, which is an early event in the initiation of apoptosis.[221] However, in *Saccharomyces cerevisiae* prion protein does not require other Bcl-2 family proteins to protect against Bax-mediated cell death.[222,223] Further investigation by Chen et al[182] revealed that the mouse PrP[C]-human IgGF$_c$ fusion protein that resulted in increased cerebellar granule cell neurite survival also increased antiapoptotic Bcl-2 levels and decreased proapoptotic Bax levels, suggesting that PrP[C] may be influencing neurite survival via a Bcl-2 family mediated mechanism. In addition, PrP[C] overexpression in a human breast carcinoma cell line (MCF-7) induced resistance to tumor necrosis factor (TNF)-α-induced cell death.[224] Overall, these data suggest that PrP[C] may be influencing neuronal survival by inhibiting Bax activation, however, the exact mechanism by which PrP inhibits Bax remains to be elucidated. Furthermore, anti-PrP[C] antibodies inducing cross linking of PrP[C] molecules triggered apoptosis of hippocampal neurons in vivo.[215] Whether this apoptosis is the result of the loss of a cell survival signal from PrP[C] or the transmission of a cell death signal was not evaluated.

Mouse Models to understand the function of PrP and its involvement in prion diseases

Several mouse models of PrP have been developed over the years and have helped further understand the function of PrP in vivo. These are discussed below and summarized in the Table.

PrP[C] knockout mice (PrP[0/0])

Prusiner[47] proposed that the agent responsible for the transmissible spongiform encephalopathies was devoid of nucleic acid and composed only of protein (PrP[Sc]). This was based largely on the resistance of the agent to numerous treatments that destroy nucleic acid, the small size constraints imposed on the agent, the inability to demonstrate nucleic acid, and the reduced infectivity induced by treatments that degrade proteins.[47] In an attempt to find more conclusive evidence to support their theory, Bueler et al[225] succeeded in disrupting one mouse Prnp allele of murine embryonic stem cells by homologous recombination with a 4.8-kilobase DNA fragment in which codons 4 to 187 of the 254-codon open reading frame were replaced by a neomycin phosphotransferase (neo) gene under the control of the *Herpes simplex* virus thymidine kinase (HSV TK) promoter. Blastocysts from C57BL/6J mice were injected with the clone and implanted into foster mothers.[225] The resulting offspring were screened with polymerase chain reaction (PCR) and Southern analysis to confirm the presence of the disrupted gene and these heterozygotes mated to generate homozygous Prnp[0/0] knockout mice.[225] The genotype of these mice was also confirmed by PCR and Southern analysis.[225] Manson et al[226] also produced a disruption of the gene. Both groups found that, contrary to expectation, the mice survived and developed normally.[225,226] These homozygous null mice (Prnp[0/0]) did not develop spongiform encephalopathy after inoculation with PrP[Sc], and did not demonstrate infectivity.[100] These findings were consistent with the protein-only hypothesis, and demonstrate that PrP[C] is crucial to the development of and transmission of the disease, but do not prove that PrP[Sc] is the actual agent. Interestingly, mice heterozygous for the Prnp

knockout expressing reduced levels of PrP[C] developed disease, but had prolonged incubation times.[227]

Prnp[0/0] mice are reported to have altered circadian rhythms and sleep patterns.[187] They may also have impaired memory retention with aging.[188] Abnormal synaptic activity involving gamma aminobutyric acid (GABA$_A$) type A receptors in the CA1 region of the hippocampus was reported by Collinge et al,[194] although no effects on synaptic function were found by Lledo et al.[195] Significant reductions in afterhyperpolarizations (AFPs) in hippocampal CA1 cells were also reported in a post-natal knockout of prion protein targeted to neurons in the adult mouse, suggesting a role for PrP[C] in regulating neuronal excitability.[166] Clearly, there is no consensus at this time, regarding the effect of PrP[C] on synaptic activity.

Prnp[0/0] mice are also reported to have demyelination in the peripheral nervous system, although no clinical signs have been noted in the Zurich strain developed by Bueler's group.[228] The clinical signs described in other knockout strains and not in the Zurich strain, are believed to be related to variable disruption of the gene in different strains.[228]

Cells from Prnp[0/0] mice also have alterations in superoxide dismutase activity (SOD-1) and therefore increased susceptibility to oxidative stress[173] and defects in copper metabolism.[167] Prnp[0/0] mice are more susceptible to seizures than their wild-type counterparts, which is also thought to be related to increased susceptibility to oxidative stress.[189] Brown et al[229] examined numerous biochemical parameters and found increased activity of nuclear factor NF-κB, increased activity of manganese superoxide dismutase, perhaps in response to decreased p53, as well as decreased Cu/Zn superoxide dismutase activity. They concluded that Prnp[0/0] mice, or cells derived from them, have increased neuronal sensitivity to oxidative stress.

Another Prnp knockout generated by Sakaguchi et al[230] led to the overexpression of a gene downstream from the Prnp gene, a PrP[C]-like protein called Doppel (Dpl), and progressive ataxia and cerebellar Purkinje cell degeneration by 70 weeks of age.[231] This phenotype is rescued by the coexpression of PrP[C].[232] Purkinje cell degeneration is not a feature of the transmissible spongiform encephalopathies (TSEs) in mice, suggesting that this Doppel-induced disease is a distinct entity and unrelated to the TSEs. Interestingly, an N-terminally truncated form of PrP, similar to Doppel, when targeted to Purkinje cells, also leads to Purkinje cell loss and is also rescued by the reintroduction of PrP[C].[233]

PrP[c] Overexpressor mice (PrP[Oexp])

In order to assess the species specificity of scrapie infectivity, Scott et al[234] and Prusiner et al[235] constructed mice expressing Syrian Hamster prion protein (SHaPrP[C]), as well as endogenous mouse prion protein (PrP[C]). Eggs were obtained from superovulated (C57BL/6 X SJL)F1/J hybrid females mated to (C57BL/6 X SJL)F1/J hybrid males. DNA constructs were made by fusing sequences of the SHaPrP promoter, a fragment of a cDNA clone encoding the open reading frame and a restriction fragment containing the SHaPrP polyadenylation signal.[234,235] The DNA constructs were microinjected into the pronuclei of one-celled mouse eggs. Weanling animals were screened for the presence of the SHaPrnp gene using Southern transfer analysis.

Mice expressing hamster PrP[C], in addition to mouse PrP[C],

became susceptible to infection with Syrian Hamster prions, to which they are normally resistant,[234] and the incubation time paralleled protein expression level.[235] When infected with mouse prions, they had a longer incubation time than non-transgenic littermates.[234] The importance of the amount of PrP[C] in the cell to the development of the disease was illustrated by the observation that transgenic mice overexpressing wild-type PrP, when infected with mouse prions, had a shorter incubation time.[236]

These findings support a direct protein-protein interaction in the propagation of the disease. Unlike their PrP[C] knockout counterparts, mice expressing wild-type PrP[C] derived from either hamsters or sheep developed spontaneous disease, in proportion to the copy number of the transgene.[237] The pathological changes described included necrotizing myopathy, demyelinating neuropathy and focal spongiform degeneration in the central nervous system (CNS).[237] Cell death via overexpression of PrP is also demonstrated by Paitel et al[238] where stably transfected cell lines overexpressing PrP[C] rendered cell lines more sensitive to caspase-3-mediated apoptosis.

These data indicate that the host PrP[C] is qualitatively and quantitatively essential for the development of the disease.

Other transgenic mice

Thus far, approximately 20 distinct mutations in the PRNP gene have been identified.[17] Partly due to the "species barrier" (i.e. difficulty in transmission of a TSE agent to a new species with a different PrP primary structure on first passage) and partly due to few studies attempting transmission, which may be a reflection of the rarity of clinical cases and therefore infectious material, brain homogenate from few of these familial mutations have successfully transmitted disease to mice. Brain homogenate from familial mutations such as E200K, M232R, P102L and D178N have successfully induced disease in mice, with variable degrees of difficulty.[239]

In the case of the human mutation P102L, the equivalent mouse mutation (P101L) has been created in transgenic mice expressing approximately eight fold normal amounts of PrP and these mice spontaneously developed neurological "symptoms" of ataxia, lethargy and rigidity.[240] Histologically, spongiform degeneration was identified in most gray and white matter structures in the cerebral hemispheres and brainstem with mild to moderate reactive astrocytic gliosis. Using 10% brain homogenate, this disease was successfully transmitted to a transgenic line expressing the same mutation at an estimated 2 fold normal levels of PrP. However, it was not transmissible to wild-type mice, even though brain extracts from human patients with the P102L mutation and human prion primary structure is transmissible to wild-type mice with the mouse primary prion structure.[239]

Similarly, in an attempt to reproduce the human familial A117V mutation, Hegde et al[128] created a transgenic mouse predominantly expressing a C transmembrane form of the prion protein, [Ctm]PrP, with Ala to Val substitutions at positions 113, 115, and 118 (PrP AV3). They were unable to demonstrate the presence of PrP[Sc] in the mice, however, these mice still developed neurological disease characterized clinically by ataxia and paresis. They described focal vacuolar degeneration in the neuropil of the gray matter, which was most pronounced in the

hippocampus and piriform cortex, however, lesions in these locations do not explain the clinical signs. Hegde et al[128] reported that increased [Ctm]PrP was noted in the single GSS brain with codon 117 mutation relative to the single control brain that they examined. However, patients with this mutation predominantly present with dementia and Parkinsonian signs, with ataxia described as a minor feature.[6] Histologically, these GSS patients have numerous amyloid plaques, occasional neurofibrillary tangles with variable vacuolation, gliosis and neuronal loss.[6] These data suggest that the transgenic overexpression of [Ctm]PrP may be associated with neurological disease in mice, however, the evidence for a link between [Ctm]PrP and codon 117 mutation GSS is less convincing.

Mutations within the Prnp gene, coding for a truncated amino terminal, were reintroduced into Prnp[0/0] mice, and resulted in granule cell loss in the cerebellum and subsequent ataxia, a phenotype that was rescued by the reintroduction of a single copy of the Prnp gene.[241] Granule cell loss is not a feature of TSEs in mice, suggesting that this truncated amino terminal form of the protein is not significant in the development of the TSEs in mice. Shmerling et al[241] speculated that these truncated PrPs may have competed with some other molecule for a ligand and disrupted the ligand's function.

Ma et al[162] also created a transgenic mouse expressing cytosolic PrP (PrP[C] lacking both the N-terminal and C-terminal signal peptides). These mice were ataxic and had cerebellar atrophy due to massive granule cell loss. Apparently, the transgene lacked the necessary enhancer element to be expressed in Purkinje cells, and so no changes were detected in these cells. Ma et al[162] proposed a unifying model for the pathogenesis of the prion diseases, suggesting that mutations and presumably the infectious agents, increase misfolding of PrP[C] triggering the cell to transport these proteins to the cytosol, where they are metabolized by the proteasome. They proposed that impaired proteasomal function could provide a mechanism for the build-up of prion proteins in the cytoplasm. Similarly, Cohen and Taraboulos[242] treated cells with cyclosporin A to inhibit peptidyl-propyl cis-trans isomerases (PPIases) and detected the accumulation of a protease-resistant "prion-like" PrP species, which was not degraded by the proteasome and accumulated in nonmembrane bound aggresomes. Cohen and Taraboulos[242] however, did not report cell degeneration or death as a consequence of their treatment. Ma et al[162] found that "very small quantities of soluble PrP are toxic". Their construct induced cell death, presumably by apoptosis, as there was no inflammation described. However, neuronal loss has not been unequivocally described in scrapie, and may not be present in cases of CJD and GSS.[53] Granule cell loss is not a feature of scrapie in mice, in fact, of the approximately 20 distinct scrapie strains recognized in mice inoculation tests, only 22L has significant cerebellar lesions, and these consist of vacuolation of the gray matter.[31] The only other mouse model of TSE that produces significant cerebellar lesions is BSE, where Purkinje cell loss, rather than granule cell loss has been described.[40] The unifying process in the spongiform encephalopathies is the loss of the ability of neurons to maintain their specialized function with cell death occurring some time later i.e. degeneration, and so a proposed process that kills neurons when very small quantities of PrP are in the cytosol rather than simply impairing

their function would not explain the observed sequence of lesions in the spongiform encephalopathies. For the cytoplasmic PrP theory of Ma et al[162] to be valid in the naturally occurring diseases, the accumulated PrP isoform would have to impair neuronal function rather than simply initiate the cell death pathway. In this respect, both the pathogenesis of the disease and the lesions described by Ma et al,[162] appear to be unique, suggesting that it is a unique disease and not part of the TSE spectrum. Moreover, Drisaldi et al[161] in contrast to Ma et al[162] found that blocking the proteasome in cell cultures does not result in retrotranslocation and increased PrP in the cytoplasm. Drisaldi et al[161] suggested that the neurotoxicity noted by Ma et al[162] was the result of increased PrP synthesis due to a side effect of the proteasomal inhibitor on the CMV promoter used by Ma et al[162] Drisaldi et al[161] hypothesize that the mechanism of PrP accumulation may be due to stabilization of transcription or translation factors or to activation of signaling pathways that impinge on transcription of the promoter. In addition, Roucou et al[155] found that PrP accumulation and passage through the ERAD pathway was not toxic in human primary neurons or human neuroblastoma cell lines.[155,243]

Another transgenic mouse was created that expressed additional octapeptide repeats, similar to a human inherited prion mutation.[120] These mice express nine additional octapeptide repeats (PG14) and develop neurological disease characterized clinically by ataxia. Histologically, there is massive cerebellar granule cell loss, PrP accumulation and astrogliosis. This disease is not transmissible to wild-type mice, and the lesion is not reported in any of the prion strains that have been typed in mice, indicating that this disease is not part of the TSE spectrum and is a disease of protein overexpression.

Table I: Mouse models and functional studies

Mouse	Molecular Effect	Lesion	Reference
PrP[0/0]	Knock out 4-187 of ORF	Altered circadian rhythm, +/- seizures	(225)
PrP[Oexp]	Addition of SHPrP cDNA	Myopathy, neuropathy, spongiform degeneration CNS	(234)
Shmerling mouse	N-terminal truncation	Cerebellar granule cell loss	(247)
Hegde Ctm mouse	Valine to alanine substitutions 113, 115, 118	Vacuolation, hippocampus, piriform cortex	(248)
PG14 mouse	9 additional octapeptide repeats	Cerebellar granule cell loss	(120)
Ma cytosolic PrP mouse	N and C terminal truncations	Cerebellar granule cell loss	(162)
Sakaguchi PrP^{-/-}	Entire coding exon knockout strategy	Cerebellar Purkinje cell loss	(230)
PrP[P102L]	8 fold overexpression of P102L	Cerebral and brainstem spongiform degeneration	**(249)**

Overall, these data suggest that the transgenic overexpression of a mutant prion protein in mice can result in a disease with neurological signs and pathological changes in the nervous system. However, none of these mutant forms of PrP have been shown to be transmissible to wild-type mice. These diseases cannot therefore be considered "transmissible" spongiform encephalopathies and are therefore diseases of overexpression of a protein in neurons that result in neuronal dysfunction and degeneration. The overexpression of other proteins may also result in neurological disease e.g. Syrian Hamster PrP,[237] α_B-adrenergic receptor,[244] neurofilament proteins (NF-L, peripherin)[245] and G-protein coupled, protease-activated receptor for thrombin.[246] Therefore, we should not be surprised that the expression of a protein above physiological levels may disrupt metabolic pathways and result in degeneration.

Conclusions

While the prion protein is the subject of numerous investigations, which have provided important scientific insight into disease and function, a number of important questions remain to be answered. Is prion protein the transmissible agent of prion diseases or is it the susceptibility factor that allows the manifestation of the disease? Are the prion protein functions important or are they part of a backup system that reiterates these functions? Are the genetic mutations truly reproducing the clinical and pathological manifestations of the disease in animal models? Clearly, much more work is required to solve these answers. Thus, future investigations promise continuing exciting results for this very unconventional protein.

Acknowledgements

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