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 Now, concerns about BSE have been replaced by a growing fear that there will be large numbers of cases of new variant CJD as a result of the consumption of BSE-infected meat.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

2

Prion Protein as Copper-Binding Protein at the Synapse

Hans A. Kretzschmar, Tobias Tings, Axel Madlung, Armin Giese, and Jochen Herms

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

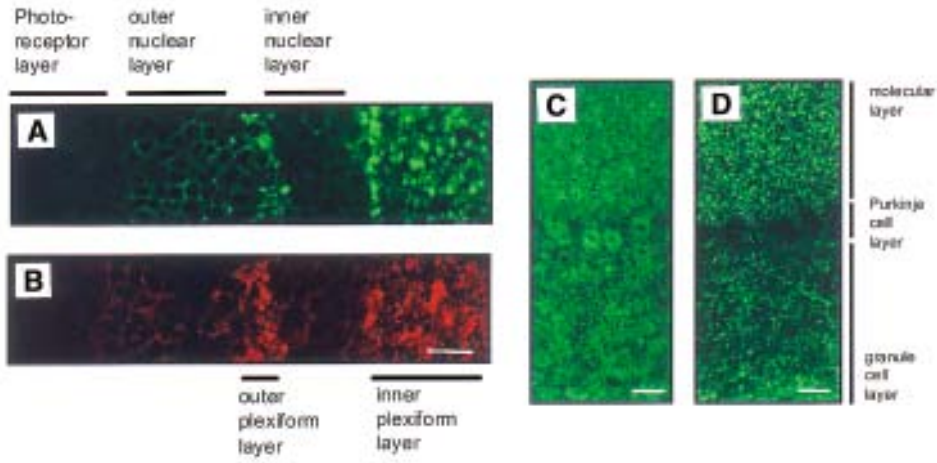

Fig. 1. Synaptic expression pattern of PrP^C in PrP^C-overexpressing transgenic mice. Laser scanning confocal images of PrPc expression in the retina and cerebellar cortex of PrP^C-overexpressing mice. Expression of PrPC (**A**) and synaptophysin (**B**) in Tg20 retina. PrP^C is strongly expressed in 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-

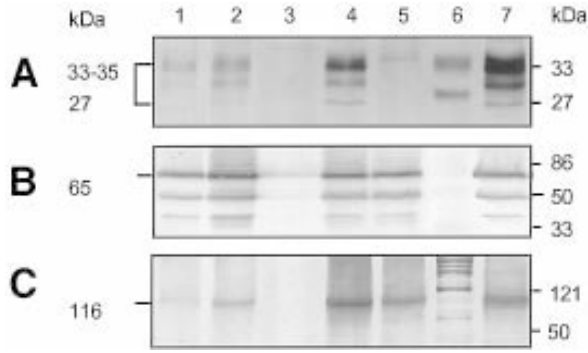

Fig. 2. Enrichment of PrP^C in the synaptic plasma membrane fraction. Preparations of the synaptic plasma membrane fraction and synaptic vesicle fractions from synaptosomes ***(54)***. Equal amounts (100 µg/per lane) of brain homogenate and various subcellular fractions from wild-type (lane 1–4), $Prnp^{0/0}$ (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

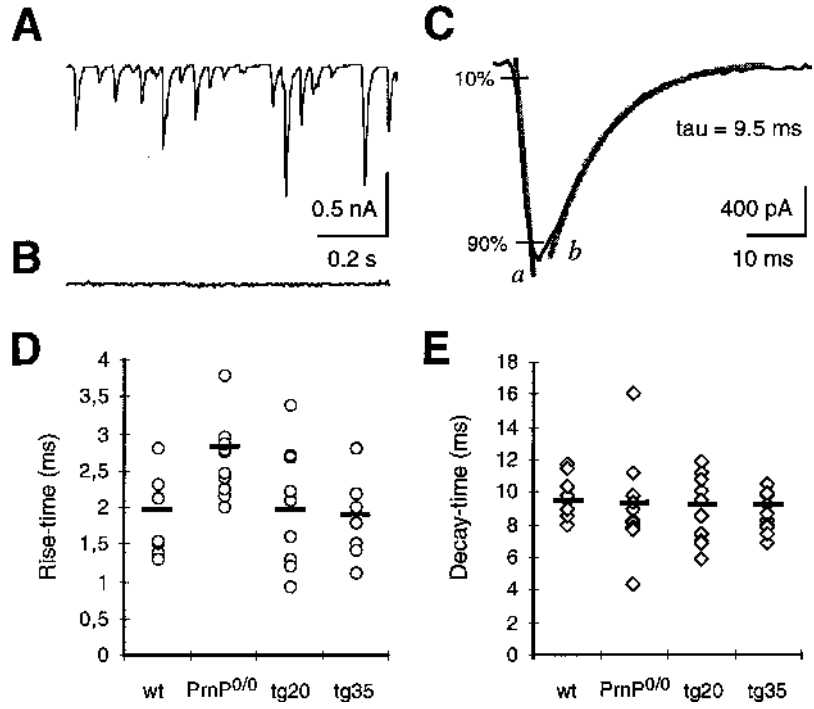

Fig. 3. Presynaptic PrP^C expression modulates the kinetics of inhibitory postsynaptic currents (IPSC). (**A**), Spontaneous IPSCs from a Purkinje cell of a 10-d-old wild-type mouse using the patch-clamp technique, as described ***(32)*** (**B**), Using the effect of 10 μM bicucullin, a γ-aminobutyric 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

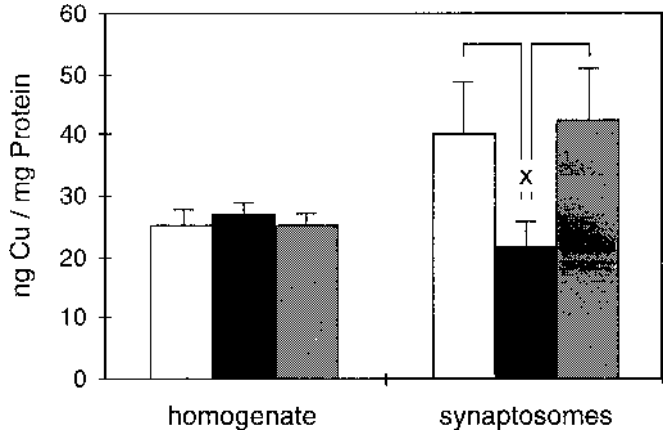

Fig. 4. Copper concentration in synaptosomes correlates with PrP^C expression. The copper concentrations in whole-brain homogenates and synaptosomal fractions from wild-type (open columns), $Prnp^{0/0}$ (black columns), and Tg20 (gray columns) mice were studied by atomic absorption spectroscopy. Shown 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 μ*M*). 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 μ*M* 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 μ*M* ***(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.

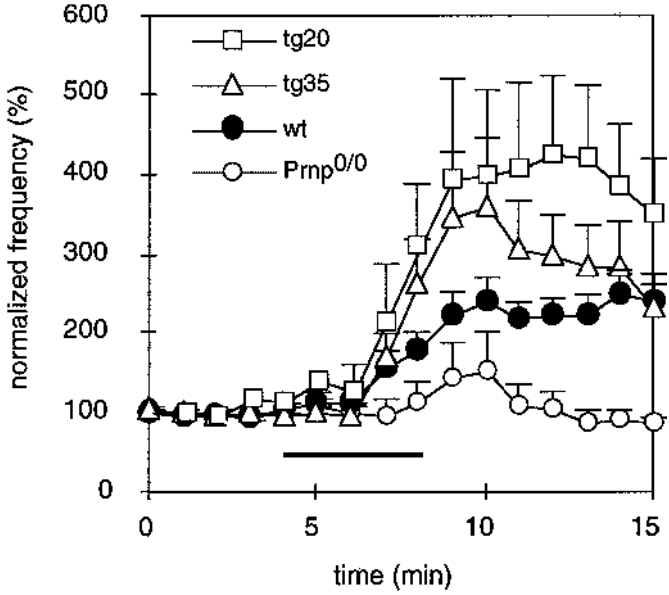

Fig. 5. Enhancement of inhibitory synaptic activity by hydrogen peroxide is related to the amount of PrP^C at the presynaptic plasma membrane. Effect of 0.01% H_2O_2 on the frequency of inhibitory postsynaptic currents in the different mouse lines. Each point represents the mean ± SEM sIPSC frequency 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

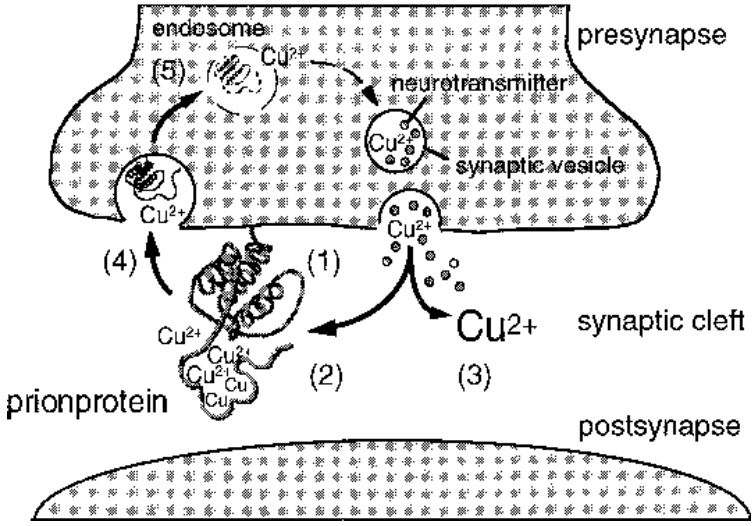

Fig. 6. Hypothetical model showing a possible function of copper binding by PrP^C at the synaptic plasma membrane. The prion protein is attached to the presynaptic plasma membrane (1) ***(23)***, where its N-terminal moiety (2) binds free copper that is released into the synaptic cleft with synaptic 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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