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The Role of the Prion Protein in the Molecular Basis for Synaptic Plasticity and Nervous System Development

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Abstract The cellular prion protein (PrP^C) is found prominently at the synapse. However, its role at the nerve termini and elsewhere is unknown. Here we discuss research presented at the 2005 International Institute for Complex Adaptive Matter (I2CAM) first Annual Amyloid Conference that provides insight into the role of synaptic PrP^C. The prion protein can interact and facilitate copper uptake at the synapse, is presumed to oligodimerize to facilitate putative cell-cell adhesion, and it transports toward the synapse by fast microtubule-based anterograde transport. While PrP^C appears to be involved in all these processes, the mechanisms of PrP^C function in each of them remain unclear. A role for PrP^C in these distinct processes suggests a complex role for this protein at the synapse. Unraveling PrP^C function will likely entail employing combined approaches that take into account its possible multifaceted functions.

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Introduction

The cellular prion protein (PrP^C) has been identified as an indispensable component for the initiation of infectivity in transmissible spongiform encephalopathies (TSEs). However, the physiological function of this non-infectious prion isoform remains elusive. Diverse lines of evidence point to a potential role of PrP^C in cell adhesion, oxidative stress, copper uptake and cell signaling, and several studies have suggested a role for the protein in synaptic function (Collinge et al. 1994; Herms et al. 1999; Mouillet-Richard et al. 2000; Roucou et al. 2004; Vassallo and Herms 2003). In the summer of 2005, the International Institute for Complex Adaptive Matter (I2CAM) held the first annual Conference on "Protein Aggregation and Amyloid Formation in Systemic and Neurodegenerative Diseases" in Lausanne, Switzerland. Among the sessions, one focused on the putative role of the prion protein in synaptic plasticity and development. Four presentations by the authors in this section concentrated on issues relating the function of the prion protein at synapses. Here, we summarize the main issues and questions addressed in this session as they related to the following open questions: What is the normal function of PrP^C? What is the evidence for PrP^C's role in synaptic function? What is the role and mechanism/s of axonal transport of PrP^C in the peripheral and central nervous systems?

The presenters in the first part of the session aimed to summarize the relationship between the prion protein and the synapse and gave an overview of new data that support

the status of PrP^C as a protein with a synaptic function. During the summary, Ken Moya formerly from CEA (Orsay, France) and Sylvain Lehman from the Institute of Human Genetics (CNRS, Montpellier, France) noted that, consistent with its role at the synapse, studies by a number of labs have shown that PrP^C is localized pre- and post-synaptically, at central synapses, and within the neuroplasm of presynaptic boutons in several brain regions (Collinge et al. 1994; Fournieret et al. 1995; Sáles et al. 1998; Mironov et al. 2003). More distinctively, PrP^C is predominantly found in the synaptic plasma membrane and in synaptic vesicles (Chishti et al. 1997; Herms et al. 1999), and electron microscopy (EM) studies localize it in organelles including endosomes, clathrin coated pits, and the Golgi apparatus (Laine et al. 2001; Mironov et al. 2003; Bailly et al. 2004). In these organelles, PrP^C is highly expressed and attached to the cell membrane via a glycosyl phosphatidylinositol (GPI) anchor (Stahl et al. 1987; Baldwin 2005). Cell surface PrP^C interacts with copper and also a number of proteins including cell adhesion molecules such as vimentins, the laminin receptor and N-CAMs, suggesting that PrP^C might function in cell adhesion, ligand uptake, or in transmembrane signaling (Mange et al. 2002; Santuccione et al. 2005). Thus, the localization of PrP^C at the synapse and its association with various proteins and molecules including copper, is consistent with a putative role in synaptic transmission and/or in regulation of presynaptic copper concentration (Herms et al. 1999).

The Function of PrP^C: Evidence for a Role at the Synapse

The prominent presence of PrP^C at the synapse suggests that the protein plays a major role there, but its precise physiological function is not known. The N-terminus of PrP^C binds metal ions and in particular copper with high affinity (Hornshaw et al. 1995). This binding has been suggested to be relevant for the function of the protein (Brown et al. 1997). The binding of copper by PrP^C is via the octapeptide repeat region (OPR) which in humans spans residues 51–91, and it is thought to be involved in the physiological function of PrP^C (Hornshaw et al. 1995; Brown et al. 1997). A heparin binding site has been identified within the OPRs and the affinity of heparin to these sites was shown to be enhanced in the presence of copper (Gabizon et al. 1993; Warner et al. 2002). Moreover, the laminin receptor can function as a receptor for PrP^C in the presence of heparan/sulfate (Hundt et al. 2001; Gauczynski et al. 2001). Recently, it has been suggested that PrP^C interacts with the laminin receptor that is found in the perineuronal nets consisting of proteoglycans and glycosaminoglycans that surround particular neurons (Baloui et al. 2004). PrP^C has also been suggested to be involved in the regulation of copper transport (Kretzschmar et al. 2000; Brown et al. 1997), and PrP^C that is released from synapses may regulate copper concentration in the synaptic cleft (Brown 1999). High concentrations of copper are able to modify the endocytosis of PrP^C through re-uptake into the presynapse (Kretzschmar et al. 2000). Taken together, these reports suggest complex interactions at the cell membrane between these proteins and copper.

Ralph Zahn from Alicon (Zürich, Switzerland) presented work on the use of nuclear magnetic resonance (NMR) spectroscopy and dynamic light scattering to investigate the role of pH changes in the structure of PrP^C, and how these changes in structure might impinge on the function of this molecule at the synapse. Although NMR solution structures of recombinant forms of intact human (Zahn et al. 2000), bovine (Lopez Garcia et al. 2000) and murine (Riek et al. 1997) PrP^C have been described previously, these have all been obtained under acidic conditions (at pH 4.5). Thus, with the exception of a NMR structure of a C-terminal fragment corresponding to the globular domain of human prion protein, hPrP(121–130) which was determined at pH 7.0, there is no detailed structural information of the intact PrP^C protein available at physiologically relevant pH.

Zahn's group produced four recombinant PrP^C polypeptides for their studies: the mature form of the human prion protein containing four OPRs, hPrP(23–230); a polypeptide containing a single OPR, hPrP(81–230); a polypeptide completely lacking OPRs, hPrP(90–230); and a polypeptide containing only the well–structured globular PrP domain, hPrP(121–130). Zahn's group found that at pH values between 6.5 and 7.8, i.e., pH values at the cell membrane, the OPRs in the recombinant human prion protein, hPrP (23–230) are structured. At the pH value of 6.2, the NMR structure of the OPRs revealed a reversible oligomerization state that was pH-dependent. Within these OPR oligomerization or aggregation motifs the repeats reveal segments that adopt loop and β-turn-like structures. Comparison of these small aggregation motifs with the crystal structure of an aggregation motif binding to copper indicates that copper binding further induces a structural conformational change that might regulate PrP^C aggregation. In addition, Zahn's studies identified the OPRs' binding sites that are likely responsible for aggregation to span residues 60–91 of PrP^C rather than regions flanking these residues. Whether the aggregation motifs are dimeric or oligomeric in nature is not known, but oligomerization of OPRs is consistent with yeast two-hybrid data provided by other investigators showing that OPRs interact with one another (Hundt et al. 2003). Zahn's work reveals the significance of the in vitro functional presence of homo-oligomeric aggregation motifs in PrP^C molecules. Thus, the aggregation motifs of PrP^C at the cell surface might interact

with corresponding motifs of PrP^C on adjacent cells, potentially mediating intercellular contacts and suggesting a role for PrP^C in putative cell–cell adhesion. Alternatively, this homo-oligomeric aggregation of OPRs may serve to concentrate PrP^C molecules at the cell surface (presumably in lipid rafts), and thus stimulate the endocytosis of PrP^C into presynaptic vesicles (Pauly and Harris 1998; Perera and Hooper 2001). Work from Sylvain Lehmann's lab (see below) has already demonstrated a potential involvement of PrP^C in cell adhesion (Mange et al. 2002). Lehmann's data and results from the Zahn lab, together with the prevalent presence of PrP^C at synapses implicates this protein in intercellular communication through its OPRs, and show a probable role for copper in modulation of these interactions which could be occurring at axonal and/or dendritic sites.

Also in regards to the possible function of PrP^C at the synapse, Sylvain Lehmann presented work on the role of PrP^C in copper uptake. His lab applied radioactively labeled copper to cultured cells and showed that copper was associated with PrP^C on the cell membrane but was not delivered to the inside of the cell. Furthermore, the amount of copper bound to the cells increased in proportion to the amount of PrP^C expressed by the cells (Rachidi et al. 2003a). These data suggests significant interactions between copper and PrP^C. However the stimulation of PrP^C internalization by copper may not be selective since addition of exogenous glycosaminoglycans also induces PrP^C internalization. Further studies in Lehmann's group to investigate copper's binding capacity consisted of the application of radioactively labeled copper to a cell culture model that was infected with prions (Rachidi et al. 2003b). Prion-infected cells treated with copper showed a marked decrease in copper binding capacity as compared to non-infected cells. This is consistent with the idea that changes in conformation from PrP^C to a proteinase-K resistant form in these prion-infected cells resulted in the preferential association of the resistant form with zinc and manganese rather than copper (Wong et al. 2001). These results suggest that the prion protein modulates copper content but that after conversion to a proteinase-K resistant form, the protein is associated with metal ions differently than the normal protein which might play a significant role in prion disease neuropathology.

Lehmann's lab also investigated the role of PrP^C in cell adhesion (Mange et al. 2002) and reported that neuroblastoma (N2a) cells in culture transfected with PrP^C and expressing the prion protein at high levels aggregated much more than non-transfected cells. Moreover, previous studies have shown that cell adhesion that is regulated by integrins, cadherins and the immunoglobulins superfamily of proteins, is cation-dependent (particularly Ca^{2+} and Mg^{2+}-dependent; Aplin et al. 1998; Buckley et al. 1998). However in the adhesion of N2a mediated by PrP^C, the presence of copper or other cation chelators had no significant effect, while the addition of a polyclonal antibody raised against residues 45–66 of the N-terminus of the prion protein significantly inhibited N2a cell aggregation. Data from the Lehmann lab thus suggest that PrP^C promotes cell adhesion interactions that are copper-independent and that this might involve interactions with the N-terminal part of the protein. Interestingly, the antibody used by Lehmann's group to block cell aggregation was raised against the part of the prion protein that contains at least part of the OPRs. These are the same motifs in PrP^C that Ralph Zahn's lab has implicated in homo-oligomerization interactions that possibly mediate cell-cell adhesion (see above). Alternatively, binding of PrP^C to the laminin receptor, and/or N-CAMs might mediate these cell–cell interactions (Santuccione et al. 2005).

To further study the function of PrP^C, Lehmann presented a new experimental model based on the isolation of neural stem cells from mouse embryos. Using diverse differentiation protocols, Lehmann's group isolated neuronal precursor cells that divide constantly and that are nestin positive. These neuronal precursors can be further differentiated into astrocytes, oligodendrocytes and neurons that can establish synapse-like contacts. By changing the levels of PrP^C expressed in these cells, the Lehmann group has observed significant effects in neurite outgrowth. This emerging experimental model could provide a more relevant system for the study of neurodegeneration and neurotoxicity than current cell culture models that are not faithful mimics of in vivo situations.

Finally, Lehmann's group is in search of synaptic proteins with prion-like behavior. While in mammalian prion diseases the PrP^C isoform is converted to a pathological isoform, a similar non-pathogenic conversion phenomenon has been also identified for a number of yeast proteins and lately in *Aplysia californica*, the California sea hare (Lindquist 2002–2003; Wickner et al. 2004). One of these proteins, CPEB, has been recently implicated in long-term memory function in *A. californica* (Si et al. 2003; Shorter and Lindquist 2005). This is based on the role of CPEB in the maintenance of memory in *Aplysia* and on its prion-like behavior when expressed in yeast. However, the mechanism for long-term memory involvement of a self-replicating prion-like synaptic protein is not very clear. To further address this putative role in memory maintenance, several groups, including Lehmann's, are trying to identify other synaptic proteins that might have prion-like properties via *in silico* analyses. By studying the biochemical properties of such proteins, these groups might be able to decipher mechanisms and functions of these prion-like proteins at the synapse, including a potential role in the maintenance of long-term memory.

PrP^C in Axons and at the Synapse: Localization and Axonal Transport

Studies on the detailed localization of PrP^C in the brain have produced data that show inconsistencies probably because of the diversity of particular techniques employed (for a review see Harris et al. 2003). To name a few, these approaches include immunohistochemical methods that have described somatic expression of PrP^C in neurons with little or no signal at the neuropil (DeArmond et al. 1987; Piccardo et al. 1990; Safar et al. 1990; Bendheim et al. 1992; Ford et al. 2002), or quantitative studies of the ultrastructural localization of PrP^C using high-resolution techniques that combine immunofluorescence and Immunogold labeling of cryosections at light microscopy and EM levels (Mironov et al. 2003). The latter technique was used to evaluate the distribution of PrP^C in brain with particular emphasis on the hippocampus. With this technique, Mironov et al. (2003) found gold particles in a number of subcellular locations including the endoplasmic reticulum (ER), Golgi complex, endosomes and lysosomes, tubules, vesicles without coat, clathrin-coated vesicles, plasma membrane, mitochondria, and nucleus among others. In addition, these latter studies showed a distinctive presence of PrP^C in the neuropil of axonal and dendritic membranes as well as in synaptic and perisynaptic membrane profiles. More recent immunogold electron microscopic studies reported PrP^C localization mainly at presynaptic sites, and notably at asymmetric excitatory synapses in several brain regions (Bailly et al. 2004).

At the Amyloid I2CAM conference, Ken Moya presented his contribution to detailed subcellular studies of the localization of PrP^C. In adult hamster brains, Moya's immunohistochemistry studies revealed intense immunoreactivity for PrP^C in the subplate and cortical layer 1. The former contains axons growing into or out of the cortical plate while the latter has the earliest developing synapses. In the striatum, fiber fascicles were densely labeled with PrP^C, and the protein was also found on the surface of living axons (Sáles et al. 2002). Moreover, an important contribution of Moya's work has been his findings that the cellular localization of PrP^C undergoes important changes during development. For example, in hamsters at post-natal day 1, PrP^C is found largely localized in axons in the cortical plate, hippocampus and anterior commissure, while by post-natal day 11 staining of PrP^C at the neuropil is more diffuse and the anterior commisure is devoid of PrP^C altogether. At post-natal day 19, there is only diffuse PrP^C distribution in the neuropil. Complementing the developmental localization studies Moya reported that levels of PrP^C change with brain maturation. The levels of three commonly found PrP^C isoforms (36, 33 and 28 kDa species, corresponding to di-, mono-, and un-glycosylated forms respectively), appear to peak 2 weeks after birth but decrease thereafter (Sáles et al. 2002). Using a two-site immunometric assay to quantify PrP^C levels in different brain regions Moya's group reported at least three developmental profiles. The first profile shows a large increase of PrP^C during the first couple of weeks after birth in the olfactory bulb and the hippocampus, two structures that are known for their ongoing plasticity throughout the life of the animal. The second profile shows a moderate increase in PrP^C expression early after birth in the cortex and superior culliculus, with a peak in expression at post-natal day 21 and then a decrease. Both of these latter structures undergo exuberant axonal growth and synaptogenesis postnatally followed by regressive developmental events where retraction of projections and synapses occur. Thus, the decrease in PrP^C expression after a peak might reflect these regressive events. In structures in which there is comparatively less postnatal synaptic plasticity such as the brain stem, the levels of PrP^C remained lower and changed less during development. Thus, Moya's work shows some parallels between increased PrP^C expression in regions of the brain that are undergoing active axonal growth and synaptogenesis, while more moderate amounts of PrP^C are detected in regions know for lower levels of synaptic plasticity. Moya's data showing developmental regulation of PrP^C expression during CNS formation suggests a role of the protein in neuronal development and synapse formation.

The presence of PrP^C at the presynapse suggests that this protein is transported along the axon after it is produced in the cell body. Two speakers in this session presented evidence for the axonal transport of PrP^C and provided some discussion on the possible roles of transport in PrP^C function at the synapse. By using a test section accumulation paradigm, Ken Moya showed that at the whole nerve level, PrP^C moves along sciatic nerve axons in anterograde and retrograde directions (Rodolfo et al. 1999; Moya et al. 2004). Sciatic nerves are composed primarily of sensory and motor neuron axons, but do not contain neuronal cell bodies. Twenty-four hours after nerve cut, the segments proximal and distal to the cut were analyzed for PrP^C and compared to a basal segment. The proximal and distal segments contain proteins that accumulate as they travel in the anterograde and retrograde directions, respectively, in addition to stationary proteins, while the basal segment contains stationary proteins. Using a two-site immunometric assay, the PrP^C signal in the proximal was significantly increased showing an anterograde accumulation of the protein. The signal was also increased, although to a lesser extent in the distal segment demonstrating a retrograde accumulation of PrP^C. Moya then showed that the PrP^C isoform being transported along axons corresponds to a higher molecular weight doublet form that runs at approximately 38 kDa in size that is higher than the bi- and

mono-glycosylated forms found in the basal segment which have a size of 36 and 33 kDa, respectively and which appear to be either stationary or slowly transported. Thus, it appears that a novel form of the prion protein is traveling down axons and that the main difference between anterograde versus stationary moving PrP^C is a difference in the glycosylated state of the protein.

Because PrP^C is localized in elongating axons during development and in the adult brain, Moya's lab examined whether the growth state of axons influenced the axonal transport and expression of PrP^C. They analyzed the protein in the peripheral nerve during axon regeneration after nerve crush (Moya et al. 2005). There was a significant decrease in the amount of PrP^C in the basal segment up to 3 days after the crush, which is consistent with the nerves of the crush site containing mostly degenerating axons and debris at this point in time. Ten days post-crush, when regenerating daughter axons had visibly advanced, levels of the prion protein were twice as high as those shown in non-crushed control animals. Twenty days after the crush, at the time that the regenerating nerves reach their target, PrP^C levels were equal to those in control animals. In contrast, the axonally transported PrP^C reached a peak at 20 days post crush. By day 50 after the crush, the animals had recovered limb function and the levels of PrP^C on the proximal and basal segments were similar in crushed and non-crushed control animals. In summary, PrP^C does not seem to be transported along axons early after nerve injury, but as the nerves regenerates, there is an increasing amount of prion protein traveling down axons. By the time the daughter axons reach the target and presumably start making synapses, they found the largest increase of axonally transported PrP^C forms (the 38 kDa higher molecular weight isoform). These results show that (1) PrP^C is increased during nerve regeneration, and (2) there is an increased in anterogradely transported PrP^C during regeneration. Moya noted that there is evidence that PrP^C undergoes a glycomodification that adds a HNK1 epitope to the molecule (Voshol et al. 1996; Schachner and Martini 1995). This modification has been previously implicated in cell–cell adhesion, cell recognition and guidance and thus Moya's observations might suggest a possible role for PrP^C as a guidance or adhesion molecule.

But what are the mechanisms of axonal transport of the prion protein? Much of the intracellular transport in cells is mediated by molecular motor proteins, such as kinesins and dyneins, which 'track' along polarized microtubule paths in plus- and minus-end directed routes, respectively (Guzik and Goldstein 2004; Hirokawa and Takemura 2005). Sandra Encalada from the University of California, San Diego (USA) presented a fluorescent labeling assay for observing PrP^C axonal transport in living primary mouse hippocampal cultured cells. In this system, wild-type and hippocampal cells from mice homozygous for a mutation in kinesin were transfected with a YFP-PrP^C fusion construct. PrP^C was observed traveling along single axons in 'puncta'-like structures in anterograde and retrograde directions. Preliminary analysis of the number of PrP^C puncta shows that the majority (~55%) of PrP^C puncta are stationary and approximately 35 and 10% travel in anterograde and retrograde directions, respectively. In kinesin-deficient cells the number of stationary particles increases to over 80%, with a concomitant significant decrease in the number of particles traveling anterogradely and retrogradely. These preliminary data suggest that kinesin might be involved in the transport of PrP^C down the axon. This kinesin is a subunit of Kinesin 1 (or conventional kinesin) motor protein that is thought to be involved in cargo attachment and/or regulatory functions of the protein, while the kinesin heavy chain is involved in the actual ATP-dependent motor activity (Rahman et al. 1998). Encalada noted that while there is some accumulation of PrP^C at the synapse as observed in this transport assay, the nature of the PrP^C puncta is not known and the function of the role of PrP^C transport toward and back from the synapse is not clear. Many questions regarding transport of PrP^C remain unanswered. Mainly, why is PrP^C transported toward the synapse? Once there, does it turn around or is it being degraded at the synapse? Do the prion protein molecules that travel from the synapse toward the cell body represent a different subgroup of PrP^C, and do they have a function in the cell body? Is what is traveling retrogradely being captured by the synapse from neighboring cells?

In summary, this section of the Amyloid I2CAM meeting provided us with a significant overview of some of the important pieces of evidence that suggest a role for PrP^C at the synapse. Thus, the work presented by the speakers in this session emphasized localization studies of PrP^C that clearly show expression of this protein at the synapse and a positive role of the prion protein in neurite outgrowth, and differentiation. Ultrastructural NMR and dynamic light scattering analyses or PrP^C also suggest that PrP^C might have a role in cell–cell adhesion via newly identified homodimerization domains that can serve as intercellular zippers and could facilitate not only interactions but might also provide a venue for intercellular communication and the sharing of signals between cells. These results are in agreement with cell adhesion studies that show that PrP^C overexpression enhances aggregation of cultured cells. While the role of copper in cell aggregation is not obvious, copper appears to enhance the homodimerization of PrP^C aggregation motives and has been shown to be involved in PrP^C internalization. Furthermore, the prion protein is actively transported up and down axons. In the presence of axonal injury, PrP^C was shown to be preferentially targeted toward newly regenerated axons, potentially

playing an important role in axonal growth as an adhesion molecule. The axonal transport of PrP^C might be accomplished via its interactions with molecular motors. In particular, kinesin might be involved in taking PrP^C in an anterograde direction from the cell body toward the synapse. Finally, in this session a new experimental paradigm has been introduced for the study of PrP^C expression and function. Mainly, the use of neural precursor cells and their neuronal differentiated progeny are now being used to explore further the function of PrP^C. These and similar systems could serve to more faithfully recapitulate the neuron environment and thus provide in the future a relevant model system for the study of the role of PrP^C in neurodegeneration and neurotoxicity.

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