Localizing the cellular prion protein (PrPC) in the brain is necessary for understanding the pathogenesis of prion diseases. However, the precise ultrastructural localization of PrP\textsuperscript{C} still remains enigmatic. We performed the first quantitative study of the ultrastructural localization of PrP\textsuperscript{C} in the mouse hippocampus using high-resolution cryoimmunogold electron microscopy. PrP\textsuperscript{C} follows the standard biosynthetic trafficking pathway with a preferential localization in late endosomal compartments and on the plasma membrane of neurons and neuronal processes. PrP\textsuperscript{C} is found with the same frequency within the synaptic specialization and perisynaptically, but is almost completely excluded from synaptic vesicles. Unexpectedly, PrP is also found in the cytosol in subpopulations of neurons in the hippocampus, neocortex, and thalamus but not the cerebellum. Cytosolic PrP may have altered susceptibility to aggregation, suggesting that these neurons might play a significant role in the pathogenesis of prion diseases, in particular those mammals harboring mutant PrP genes.

*Key words:* prion protein; hippocampus; immunogold; localization; membrane; cytosolic

**Introduction**

The cellular prion protein (PrP\textsuperscript{C}) is a cell-surface glycoprotein anchored by a glycosylphosphatidylinositol (GPI) moiety (Stahl et al., 1987). PrP\textsuperscript{C} is expressed throughout the brain, particularly in neurons (Kretzschmar et al., 1986; Moser et al., 1995) and to a lesser extent in extraneural tissues (Bendheim et al., 1992; Ford et al., 2002). In prion diseases, PrP\textsuperscript{C} is converted to an abnormal, conformationally altered isoform (PrP\textsuperscript{Sc}), which subsequently accumulates in the brain and results in extensive neurodegeneration with an inevitably fatal outcome (Prusiner, 1996). Therefore, localizing PrP\textsuperscript{C} in the brain is an important step in understanding the biology of the normal protein and mapping changes in models of experimental prion diseases.

The precise localization of PrP\textsuperscript{C} remains enigmatic because of conflicting data obtained using different techniques. Immunohistochemical studies described a somatic expression of PrP\textsuperscript{C} in neurons with no signal or only a minor signal in the neuropil (DeArmond et al., 1987; Piccardo et al., 1990; Safar et al., 1990; Bendheim et al., 1992; Verghese-Nikolakaki et al., 1999; Ford et al., 2002). However, it was not determined whether PrP\textsuperscript{C} was luminal [for example, in the endoplasmic reticulum (ER) or Golgi] or cytosolic. Data obtained using free-floating section immunohistochemistry (Sales et al., 1998; Haeberle et al., 2000; Moya et al., 2000) and immunoelectron microscopy (Fournier et al., 1995, 2000) indicated PrP\textsuperscript{C} localization in the neuropil with a synaptic membrane prevalence. However, data obtained from synaptosomal preparations (Herms et al., 1999) and a recent ultrastructural study on the distribution of PrP\textsuperscript{C} in the cerebellum (Laine et al., 2001) have favored a predominantly plasma membrane localization of PrP\textsuperscript{C} with no expression on synaptic vesicles or in the cytoplasm. These contradictory findings probably reflect the peculiarities inherently associated with pre-embedding techniques. Many immunoelectron microscopic procedures may result in a destruction of cellular membranes, possibly leading to an artificial redistribution of GPI-anchored proteins within the membrane (Griffiths, 1993).

Two recently published studies on the effects of proteasome inhibitors on PrP\textsuperscript{C} degradation and expression of cytosolic PrP suggested that cytosolic localization of PrP is sufficient to induce neurodegeneration (Ma and Lindquist, 2002; Ma et al., 2002). It is notable that under the experimental conditions of these studies, PrP becomes insoluble and acquires partial protease resistance.

These uncertainties regarding the precise subcellular localization of PrP\textsuperscript{C} therefore encouraged us to perform the first quantitative study of ultrastructural PrP\textsuperscript{C} localization in the mouse brain. We used a sensitive, high-resolution detection method combining immunofluorescence and immunogold labeling of 500 and 60 nm cryosections at light and electron microscopy levels, respectively. The method uses glutaraldehyde for both optimal fixation and preventing migration of GPI-anchored proteins and circumvents the need for alcohol dehydration. Because of its potentially important role in the pathogenesis of prion diseases (DeArmond et al., 1987; Taraboulos et al., 1992a), we focused on the localization of PrP\textsuperscript{C} in the hippocampus and show for the first time quantitative data of PrP\textsuperscript{C} distribution at the ultrastruc-
tural level throughout the CA1 and dentate gyrus areas. We localized PrP C on all biosynthetic and endocytic transport membrane structures of hippocampal neurons, but almost no PrP C was found in synaptic vesicles. In addition, we discovered a subset of neurons in which PrP is located predominantly in the cytosol. These cells did not show any obvious signs of neurodegeneration but may have important implications in the pathogenesis of prion diseases.

Materials and Methods

Animals and preparation of tissue. Brain tissue was obtained from 22 mice at ~12 weeks of age from four different mouse lines with the FVB background: (1) wild-type (wt) mice, (2) PrP-ablated (Prnp / ) mice (Büeler et al., 1993), (3) transgenic 4053 mice overexpressing mouse PrP C (Telling et al., 1996), and (4) transgenic 3045 mice overexpressing hamster PrP C (Telling et al., 1996). According to PHS–NIH Guide for the Care and Use of Laboratory Animals, the mice were deeply anesthetized with Nembutal and perfused transcardially, first with PBS plus heparin (1 μ/ml) for 90 sec, then with PBS for 1 min, and finally with one of the following fixatives for 5 min: 2% paraformaldehyde (PFA) in PIPES-HEPES-EGTA-magnesium (PHEM) buffer (25 mM HEPES, 10 mM EGTA, 60 mM PIPES, 2 mM MgCl₂, pH 7.2) (four B4053 mice); 2% PFA–0.2% glutaraldehyde (GA) in PHEM buffer, pH 7.2 (four B4053 mice, four wt mice, two A3045 mice, two Prnp / mice); periodate-lysine-paraformaldehyde (PLP) fixative (McLean and Nakane, 1974) with a final concentration of 2% PFA (four B4053 mice); and 2% PFA–0.2% GA in PHEM buffer, pH 7.2 (two B4053 mice). After perfusion, the brains were collected, postfixed in the same fixative for 1 hr at 4°C, washed in PHEM four times, and stored at 4°C in 0.5% PFA in PHEM buffer, pH 7.2.

Reagents and antibodies. PrP-specific recombinant antibody fragments (Fabs) D13, D18, R1, R2, E123, and E149 were derived from phage libraries and have been characterized thoroughly (Peretz et al., 1997; Williamson et al., 1998; Leclerc et al., 2001; Peretz et al., 2001). SAF32 and 8H4 monoclonal antibodies were gifts from Dr. H. Axelrad (Faculty of Medicine, Pitié-Salpêtrière, Paris, France). An aliquot of Fab D18 was conjugated to Ultraspan gold particles (0.8 nm; Aurion, Wageningen, The Netherlands) to allow increased penetration into the cryosections and circumvent labeling artifacts caused by a cross-reaction with immunoglobulins in the tissue. R-GENT SE-EM and R-GENT SE-LM silver enhancement kits were purchased from Aurion. The relative distributions of labeled PrP C were determined by counting gold particles over plasma and intracellular membranes of selected hippocampal cells. We estimated the membrane (gold per micrometer) and area (gold per square micrometer) labeling density on micrographs with a final 32,000× magnification by using point and intersection counting with a line and point lattice (10 mm distance) overlay as described by Weibel (1979) and Griffiths (1993). Gold particles located ≤20 nm from a visible membrane structure were assigned to that structure. The distance of 20 nm was chosen on the basis of the distance constraints of immunolabeling described below.

We used two labeling protocols on ultrathin cryosections. The first included the application of a murine Fab fragment, followed by a rabbit anti-mouse anti-Fab IgG and protein A coupled to a 10 nm gold particle. According to Griffiths (1993) and Amit et al. (1986), the length of an IgG molecule in projection is ~8–10 nm, and a Fab fragment is ~5 nm. The diameter of the protein A-gold (10 nm) complex is ~12–13 nm, but because we measure the distance to the center of the gold particle, we should consider only its radius (6–7 nm). In summary, the complex consists of a Fab, IgG, and protein A-gold and has a projection of 19–22 nm. Therefore, we used a ~20 nm radius to assign gold particles to a specific structure on the sections.

The second labeling protocol involves the application of recombinant Fab fragments directly conjugated with UltraSmall gold particles (0.8 nm) and the subsequent silver enhancement for better visualization. In this case, the distance between the antigen and the center of the gold particle is ~6 nm. The silver enhancement procedure deposits silver around the gold particle without preferential orientation, practically isotropic. Therefore, the position of the enhanced gold particle (10–15 nm in diameter) will precisely indicate the localization of the antigen of interest.

We analyzed cells taken from a vertical strip running through CA1 from the stratum oriens to the hilus of the dentate gyrus. This included...
cells from the pyramidal cell layer and hilus, as well as dentate granule cells. Ten random pictures with good ultrastructural preservation were taken from each area on sections from 18 grids made from three animal samples (six grids for each sample). The gold particles were counted in the following subcellular structures: endoplasmic reticulum (including the nuclear envelope), Golgi complex, endosomes and lysosomes, tubules and vesicles without definite coat, clathrin-coated vesicles and pits, plasma membrane, mitochondria, and nucleus. Immunogold labeling on mitochondria was treated as background labeling, because PrP–C has never been observed on these organelles by either biochemical or morphological methods by us or by others.

In addition, we analyzed the distribution of gold particles in the neuropil of the strata radiatum, oriens, and molecular of the dentate gyrus, on membrane profiles in dendrites and axons, and on membranes of synaptic and perisynaptic profiles. Each class was further subdivided into the plasma membrane, internal transport vesicles, spines and endosomes (only for dendrites), mitochondria, and myelin sheaths (only for axons). Membranes of synaptic complexes were classified into synaptic vesicles, synaptic specialization (which included the two closely opposed membranes in the synapse), and the presynaptic and postsynaptic membrane (which include the membranes outside the synaptic specialization region). For our cryosections, we used the same standard criteria for subcellular structures in brain cells as those used in epon sections (Peters et al., 1991). Unidentified membrane compartments were not taken into account, because they did not show substantial labeling and represented only ~5% of all cellular membranes.

To test if the labeling for PrP was not random, we calculated the relative labeling index (RLI) according to Mayhew et al. (2002). By superimposing a test-point lattice on the electron micrographs, we generated random points (P) on cellular compartments with the point density determined by the relative size of each compartment. The number of points was normalized to the number of observed gold particles (nobs), giving a value for the expected distribution of labels (nexp). The RLI is calculated by dividing nexp by nobs. Particle distributions were compared with a χ² analysis to test whether the observed distributions differ significantly from random distributions. The partial χ² value in each row in Table 4 was obtained using the following formula: (nexp – nobs)/nobs. The total χ² value was obtained by taking the sum of the partial values. If a compartment is randomly labeled, its RLI = 1 and partial χ² = 0. If a compartment is preferentially labeled, the RLI value will be >1, and its partial χ² value will contribute a significant portion to the total χ² value.

Results
Assessment of methodology and antibodies
Four different fixatives were examined to find the optimal conditions for both ultrastructural integrity and preservation of antigenicity. We found that both PLP and 2% PFA fixatives resulted in suboptimal ultrastructure preservation for ultrathin cryosections (our unpublished data). A fixative composed of both PFA and GA gave the best results for ultrastructural integrity and antigen preservation. However, the distribution of immunolabeling was the same with all fixatives used (data not shown). Thus, we used the fixative containing 2% PFA and 0.2% GA for the immunolabeling experiments.

Raising antibodies against PrP has been difficult because of the high degree of conservation of PrP sequences between species and the inhibitory activity of anti-PrP antibodies toward lymphocytes. To circumvent this problem, we used well-characterized recombinant Fabs that recognize different parts of the PrP–C molecule (Peretz et al., 1997; Leclerc et al., 2001): E123 (residues 23–37), E149 (residues 72–86), D13 (residues 96–104), D18 (residues 133–157), and R1 and R2 (residues 225–231). These Fabs were obtained from immunized Prnp<sup>0/0</sup> mice and retrieved through phage display libraries and have been shown as reliable immunoreagents that recognize PrP–C in different experimental procedures (Williamson et al., 1996, 1998; Leclerc et al., 2001; Peretz et al., 2001). Monoclonal antibodies 8H4 (which recognizes residues 158–174) and SAF32 (which recognizes residues 52–92) were used as additional positive controls. We found no differences in labeling patterns in the murine hippocampus between these different antibodies (data not shown).

Distribution of PrP–C observed by light microscopy
To determine the precise localization of PrP–C, we used cryoprotected aldehyde-fixed tissue samples, which allowed us to make both semithin (0.5 μm) and ultrathin (60 nm) sections from the very same block in a serial manner. Thus, we had the ability to easily correlate immunolabeling at cellular and subcellular levels. We used two markers for semithin cryosections: (1) gold particles that were silver enhanced (Fig. 1A) and (2) a fluorescent dye marker (Fig. 1C). Both methods clearly showed that PrP–C was found predominantly in the neuropil in all hippocampal layers, with a higher density in the strata oriens and radiatum, moderate immunoreactivity in the stratum lacunosum-moleculare, and weak labeling in the stratum moleculare and hilus (Fig. 1A, C). No significant differences in labeling intensity were observed among the CA1, CA2, and CA3 areas of the hippocampus (data not shown). Dendrites of pyramidal cells were mostly immuno-
negative inside the profile and seen as empty profiles embedded in positive surroundings (Fig. 1A,C,D). This apparently reflected the membranous localization of PrP C [see electron microscopy (EM) study below]. Specificity of the PrP C antibodies was confirmed by the absence of staining in the hippocampus with the omission of primary antibody and tissue from a Prnp 0/0 mouse (Fig. 1B).

We consistently saw a small population of cell bodies that were intensely labeled by both the immunogold and immunofluorescence procedures (Fig. 1C–E). These cells with high PrP C content in cell bodies were found with similar frequency in three of the mouse lines (wt FVB, 4053, 3405) and were absent in Prnp 0/0 mice. These cells were concentrated predominantly in the CA1 area in the strata pyramidale and oriens with a frequency of 1–2% of all cells. Furthermore, they were practically absent from the CA3 area and occasionally seen as small groups in the dentate gyrus. In addition, a small number of cells with high PrP C labeling in cell bodies was seen in the somatosensory neocortex (layers V and VI) and ventral lateral geniculate nucleus of the thalamus (data not shown). We refer to these cells as cytosolic PrP (CPrP) cells.

We did not observe any CPrP cells in the cerebellum of any of the murine lines that we analyzed. The immunofluorescent labeling of cerebellar PrP C was concentrated in the neuropil areas of the molecular layer and not in the cell bodies (Fig. 1F). In the neocortex and hippocampus, very weak punctate labeling in the cytoplasm of cerebellar neurons reflected PrP C localization in intracellular organelles, which was confirmed by subsequent EM analysis (data not shown).

Ultrastructural distribution of PrP C in the hippocampus

To gain insight into the precise localization of PrP C at the ultrastructural level, we used the very same blocks to produce ultrathin sections immediately after cutting semithin sections from the areas of interest. Thereby we could assess identical structures at both light and electron microscopic levels. By EM, PrP C labeling in the neuropil was predominantly found on the plasma membrane of dendrites, including spines, as well as dendritic transport vesicles, endosomes, axolemma, axonal transport vesicles, and myelin sheaths. In addition, the membranes of synaptic specializations, including presynaptic and postsynaptic membranes, and of synaptic vesicles (Figs. 2B–E, 3A) labeled positively for PrP C. However, quantitative analysis showed marked reduction of labeling on the synaptic vesicle membrane (Table 1). Prnp 0/0 mice displayed no immunopositive profiles for PrP C (Fig. 2A).

Light microscopy of immunolabeled sections suggested no preferential accumulation of PrP C in the profiles of the neuropil. Being aware that PrP C has been described previously as being enriched in synapses (Sales et al., 1998; Fournier et al., 2000; Haeberle et al., 2000; Moya et al., 2000), we checked the accessibility of other synaptic proteins for immunolabeling on ultrathin cryosections. In performing double-labeling experiments, we encountered the problem of false colocalization resulting from an interaction between secondary antibodies and protein A-gold (data not shown). We circumvented this artifact by using antiprP C Fab fragments directly conjugated with UltraSmall Aurion gold particles (0.8 nm) that were subsequently enlarged by silver enhancement (Aurion). Despite a slight decrease in contrast and section quality, this method excluded artifactual effects such as...
Table 1. Quantification of PrP<sup>C</sup> labeling density on the membranes of and within dendritic, axonal, and synaptic profiles from the neuropil of the CA1 area and dentate gyrus<sup>a</sup>

<table>
<thead>
<tr>
<th>Membranous profiles</th>
<th>Stratum oriens (n = 17)</th>
<th>Stratum radiatum (n = 20)</th>
<th>Stratum moleculare (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendrites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dendritic shaft</td>
<td>1.54 ± 0.04</td>
<td>1.63 ± 0.06</td>
<td>0.62 ± 0.04</td>
</tr>
<tr>
<td>Spines</td>
<td>1.58 ± 0.1</td>
<td>1.52 ± 0.09</td>
<td>0.58 ± 0.1</td>
</tr>
<tr>
<td>Transport vesicles/tubules</td>
<td>1.08 ± 0.06</td>
<td>1.12 ± 0.09</td>
<td>0.48 ± 0.1</td>
</tr>
<tr>
<td>Endosomes/lysosomes</td>
<td>1.46 ± 0.2</td>
<td>1.38 ± 0.08</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td>Axons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axolemma</td>
<td>0.94 ± 0.09</td>
<td>0.84 ± 0.07</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td>Transport vesicles/tubules</td>
<td>0.44 ± 0.04</td>
<td>0.37 ± 0.03</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>Myelin sheaths</td>
<td>0.38 ± 0.05</td>
<td>0.35 ± 0.03</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td>Synaptic complexes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presynaptic bouton</td>
<td>1.6 ± 0.08</td>
<td>1.65 ± 0.02</td>
<td>0.82 ± 0.11</td>
</tr>
<tr>
<td>Synaptic specialization</td>
<td>1.51 ± 0.15</td>
<td>1.48 ± 0.02</td>
<td>0.79 ± 0.12</td>
</tr>
<tr>
<td>Postsynaptic bouton</td>
<td>1.64 ± 0.1</td>
<td>1.63 ± 0.12</td>
<td>0.71 ± 0.1</td>
</tr>
<tr>
<td>Synaptic vesicles</td>
<td>0.12 ± 0.012</td>
<td>0.14 ± 0.013</td>
<td>0.096 ± 0.015</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.056 ± 0.014</td>
<td>0.073 ± 0.016</td>
<td>0.04 ± 0.008</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values represent the number of gold particles per 1 μm of membrane (gold per micrometer). Mitochondria were used to assess background labeling. Results are presented as mean ± SEM.

teristic pathway organelles, such as the ER, Golgi complex, and endosomes (Fig. 6A, B). From these data, we conclude that there could be two isoforms of PrP<sup>C</sup> in these cells, one that is membrane-bound and another that is cytosolic.

We detected cytPrP with Fabs recognizing the central region (D18 and D13) (Fig. 6), N-terminal region (Est123 and Est149), and C-terminal region (R1 and R2) of PrP<sup>C</sup>. Fab D18 conjugated with UltraSmall gold gave the same pattern of labeling (Fig. 6D). Furthermore, SAF32 and 8H4 monoclonal antibodies were equally able to detect cytPrP (data not shown). This argues that full-length PrP molecules that were present in the cytosol probably bound to some factor or aggregated into multimeric complexes, which prevent diffusion into the nucleus. Theoretically, a proteasome could cut PrP molecules into fragments, which are recognizable by all applied antibodies. However, this scenario seems much less likely, because either fragments of degraded proteins are destroyed very quickly by various peptidases present in the cytosol or the peptides should be detectable in the nucleus where they are unavailable for degradation (Reits et al., 2003).

The soma and dendritic processes of cells with cytPrP receive synaptic input from other neurons (Fig. 7A). Occasionally, axonal terminals with unusually high labeling for PrP<sup>C</sup> were also found in the neuropil (Fig. 7B). We assume that these axonal terminals are derived from CPrP cells, but it remains to be proven because of the low resolution of the immunogold method in structures densely packed with membranes. Furthermore, CPrP cells were negative for GFAP, CNPase, and S100 glial cells markers (data not shown), and their morphology was different to that of glia but more closely resembled that of interneurons.

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Figure 3. Ultrastructural localization of PrP<sup>C</sup> in the neuropil of the stratum radiatum in the hippocampal CA1 area, using Fab D18 conjugated with UltraSmall gold and enhanced with a silver enhancement kit. A. Membrane-bound localization of PrP<sup>C</sup> with dendritic cytosolic free of labeling. PrP<sup>C</sup> labeling was found on the dendritic shaft, axon terminal membrane, and profiles of small processes. B. Double labeling for PrP<sup>C</sup> and VAMP2. Large particles (silver-enhanced UltraSmall gold) (arrows) represent anti-PrP<sup>C</sup> labeling on the plasma membrane of the axon terminal and dendrites; small (15 nm) gold particles indicate VAMP2 labeling of synaptic vesicles. a, Axon terminal; d, dendrite; m, mitochondria; sb, synaptic bouton. Scale bars, 200 nm.
Previous studies have shown that inhibitory interneurons may play a prominent role in the pathogenesis of prion diseases (Beliachenko et al., 1999; Bouzamondo et al., 2000). Thus, we attempted to determine whether these cells are indeed of the interneuronal GABAergic type using several different markers for particular cell populations. None of the CPrP cells were immunopositive for calbindin, calreticulin, parvalbumin, vasointestinal peptide, neuropeptide Y, somatostatin, and b-NOS (data not shown). Therefore, the specific type of neurons to which CPrP cells belong remains to be determined.

The cytosolic localization of PrP could reflect a particular state of the neuron, which may cause or result from a dysfunction of the machinery that generates GPI-anchored proteins. To check for the latter possibility, we labeled these neurons for both PrPC and another abundant GPI-anchored protein, Thy-1. In pyramidal neurons as well as CPrP cells, the labeling against Thy-1 was concentrated on late endosomes and the plasma membrane (Fig. 7C,D) without any indication of cytosolic distribution. This argues that the GPI-adding machinery functions normally in these neurons, and another factor must account for the presence of both cytosolic and membrane-bound PrP.

CPrP cells are neither necrotic nor apoptotic

Whether PrP accumulation in the cytosol reflects stress or damage to the cells is unknown. However, morphological examination of these neurons did not reveal organelle swelling (mitochondria, ER, Golgi apparatus), disaggregation of polyribosomes, or cell and nuclear membrane breaks that are clearly indicative of neuronal necrosis. Furthermore, we did not observe any apoptotic signs such as chromatin clumping, condensation of cytoplasmic content, or accumulation of autophagic lysosomes. Mitochondria displayed organized structure with well preserved cristae, inner and outer membranes. Some of these neurons had a denser cytosol than pyramidal neurons, but it was not a feature distinguishing these neurons from others. We saw a number of “dense cells” without signs of PrP accumulation in the cytosol (Fig. 6C). Moreover, many neurons with cytPrP appeared to have a normal cytosolic density, similar to other neurons. Although morphological analysis remains the “gold standard” for assessment and quantification of apoptosis (Hall, 1999), we nevertheless checked CPrP cells on semithin sections using an apoptosis detection kit (on the basis of TUNEL methodology). None of the CPrP cells appeared to be apoptotic (Fig. 8A–C).

Quantification of PrP<sup>C</sup> in the hippocampus

The use of gold particles and the good ultrastructural preservation produced by ultracryomicrotomy gave us the opportunity to quantify the distribution of PrP<sup>C</sup> in the hippocampus. All segments of dendritic membranes (dendritic shaft, spines, transport vesicles, and endocytic structures) showed approximately the same density of gold particles per unit of membrane (Table 1). The same structures in the molecular layer of the dentate gyrus had less labeling than those in the strata oriens and radiatum, matching precisely the labeling pattern observed by LM via immunofluorescence and silver enhancement. Axonal membranes had a lower gold particle density than dendritic membranes. Myelin sheaths were labeled with fewer gold particles per length of...
membrane than axolemma (Table 1). Interestingly, we found similar PrP\textsuperscript{C} concentrations on the membrane of presynaptic and postsynaptic profiles and on those within the synapse. No preferential labeling was observed within the synaptic specialization. Synaptic vesicles were labeled just above the background level determined for mitochondria, suggesting an exclusion of PrP\textsuperscript{C} from this structure.

Proteins linked to the plasma membrane by either a GPI anchor or a transmembrane domain travel along the secretory pathway. This begins with the synthesis of the protein, then translocation into the ER, and finally movement through the Golgi complex toward the final destination, the plasma membrane. At various stages along the pathway, the proteins may become concentrated. We therefore analyzed the distribution of PrP\textsuperscript{C} on various organelles and membranes that are involved with the trafficking pathway from four hippocampal cell populations: pyramidal neurons (CA1), granule neurons (dentate gyrus), hilar neurons, and CPrP cells. We quantified the distribution in the same manner as for the neuropil, counting the number of gold particles per micrometer of organelle membrane (Table 2). The data are grouped according to cell type (pyramidal neurons, granule neurons, hilar neurons, and CPrP cells) and organelles (ER, Golgi, endosomes, transport vesicles, and plasma membrane). The overall distribution of gold particles was similar in all types of cells analyzed. The lowest concentration of PrP\textsuperscript{C} was detected in the ER and Golgi complex. An increase in PrP\textsuperscript{C} concentration occurs on the plasma membrane and in late endosomes–multivesicular bodies. Our findings correlate nicely with previously published results about the folate receptor, a GPI-anchored protein in cultured cells (Mayor et al., 1998; Chatterjee et al., 2001). Labeling for cytPrP was negligible in all neurons except CPrP cells, in which the number of gold particles exceeded \(>200\) times the usual background level from the three other types of neurons (Table 3).

The statistical significance of labeling for cytPrP was assessed by the RLI (Mayorhew et al., 2002) on the basis of a comparison between the expected and observed distributions of the gold particles. The expected gold particle distribution is derived from a lattice of test points used for morphometric counting. The null hypothesis corresponds to a random distribution (RLI = 1) of the marker between compartments such as the nucleus, mitochondria, and cytosol. The RLI for PrP in the cytosol of CPrP cells attained a value of 3.12 (Table 4), which indicates preferential nonrandom labeling. The total \(\chi^2\) value (the sum of partial values) is 1864.2, indicating that the null hypothesis must be rejected (\(p < 0.001\)). The partial \(\chi^2\) values show that the most important contributor to the total \(\chi^2\) value is cytPrP.

**Discussion**

We determined the ultrastructural distribution of PrP\textsuperscript{C} in murine hippocampus using a number of recombinant Fabs generated against different parts of PrP\textsuperscript{C}, the N terminus, central region, and C terminus. In addition, we also present the first quantitative data on the localization of PrP\textsuperscript{C} in the CA1 region and dentate gyrus of the hippocampus of several lines of wt inbred mice.

We found the following: (1) PrP\textsuperscript{C} generally follows the standard biosynthetic trafficking pathway in brain neurons with prominent presence in endosomes and the plasma membrane; (2) PrP\textsuperscript{C} has a ubiquitous distribution on the neuronal plasma membrane and cellular processes without preferential accumulation at synaptic specializations; (3) PrP\textsuperscript{C} is found with the same frequency on presynaptic as well as postsynaptic membranes and within the synapse; (4) PrP\textsuperscript{C} is almost excluded from the membrane of synaptic vesicles; and (5) PrP is expressed in the cytosol in a small population of neurons in the hippocampus, thalamus, and somatosensory neocortex but not in the cerebellum.

**Localization and quantification of PrP\textsuperscript{C} in the hippocampus**

At the light microscopic level, we saw preferential PrP\textsuperscript{C} labeling of the strata oriens, radiatum, and molecular of the dentate gy-
The very low level of labeling of synaptic vesicles in our study does not deny the potential involvement of the synapse in the pathogenesis of prion diseases, as proposed by Fournier et al. (2000). The exclusion of PrPC from synaptic vesicles in our studies correlates with the morphological and biochemical data by Laine et al. (2001) and Herms et al. (1999). The presence of PrPC in all parts of the plasma membrane, whether somatic, axonal, synaptic, or dendritic membranes (with the highest concentration on the last), argues for a more general physiological function than merely a synaptic one. Various potential functions have been proposed by several authors, including oxidative stress protection (Brown et al., 2001), copper metabolism (Pauyl and Harris, 1998), signal transduction (Mouillet-Richard et al., 2000), and mediator of intercellular contacts (Rieger et al., 1999; Schmitt-Ulms et al., 2001).

The relative concentration of PrPC in organelles along the trafficking pathway observed in our study agrees well with the general theory that proteins are concentrated along this pathway en route to the plasma membrane. Here, we present quantitative morphological results on the localization and concentration of PrPC in subcellular organelles of the trafficking pathway in neurons in situ. These results are in agreement with the intracellular retention of GPI-anchored proteins in endosomal compartments (Mayor et al., 1998) and with data concerning the recycling of PrPC from plasma membranes via endosomes (Shyng et al., 1993). Reports on the concentration of PrPSc in endosomal and lysosomal structures (Caughey et al., 1991; McKinley et al., 1991; Borchelt et al., 1992; Taraboulos et al., 1992b; Arnold et al., 1995) underscore the importance of these sites for PrPC degradation and possibly PrPSc-to-PrPSc conversion.

The absence of PrP labeling in clathrin-coated structures contradicts the suggestions by Laine et al. (2001). However, their data could be attributed to either the possible diffusion of the peroxidase reaction product to nearby locations, giving false-positive results, or too low concentrations of PrPC in clathrin-coated structures, which were undetectable by our immunogold procedure. Because...
it is known that neurons do not have calveoli, we can speculate that PrP\textsuperscript{Sc} recycles through endosomes via a nonclathrin, noncalveoli pathway (Peters et al., 2003).

The almost ubiquitous distribution of PrP\textsuperscript{C} on the neuronal plasma membrane and cellular processes without a preferential accumulation at synaptic specializations suggests the absence of active retention mechanisms, allowing unhindered diffusion of PrP\textsuperscript{C} along cellular membranes. This diffusion could play a major role in PrP\textsuperscript{Sc} propagation, because it was shown that a defective fast axonal transport does not interfere with prion neuroinvasion (Kunzi et al., 2002). It is entirely plausible that PrP\textsuperscript{Sc}, which retains the GPI anchor, could physically contact PrP\textsuperscript{C} on adjacent cells or even be physically translocated to the membranes from neighboring cells (Liu et al., 2002) at sites of very close apposition to cellular membranes, including at the synapse. Additionally, the finding of significant PrP\textsuperscript{Sc} labeling in myelin sheaths points to the possible involvement of oligodendrocytes in the propagation of prion diseases.

**Neurons containing cytPrP**

Our studies revealed the existence of neurons containing cytPrP. These cells showed a very different morphology from glial cells and are negative for GFAP (an astroglial marker) and CNPase and S100 (oligodendrocytic markers). We observed synapses on cell bodies and processes as well as axonal terminals with a high PrP\textsuperscript{C} content, which probably belong to CPrP cells. On the basis of our LM and EM observations, we assumed CPrP cells to be of neuronal nature. However, we are still focusing our efforts on identifying the neuronal subtype. An antibody against GABA (the most commonly used marker for interneurons) did not give any positive labeling in cryosections. We believe that this can be explained by the fact that a very small molecule, such as GABA, is not adequately retained in cryosections, despite the use of glutaraldehyde. The additional cross-linking through an embedding medium, such as resin, may be necessary to preserve GABA localization in ultrathin sections (Bouzamondo et al., 2000), but it inevitably destroys the antigen sites for PrP\textsuperscript{Sc} antibodies (our unpublished observations).

PrP accumulation in the cytosol might depend on the circadian cycle of the cells or reflect damage and stress. However, morphological examination of these neurons did not show any organelle swelling, disaggregating of polyribosomes, or breaks in the cell and nuclear membranes that are indicative of neuronal necrosis. Furthermore, we did not find any morphological or immunocytochemical apoptotic indicators. The absence of TUNEL labeling revealed that CPrP cells do not possess breaks in nuclear DNA that are characteristic of apoptotic cells. Therefore, we conclude that these cells did not suffer any observable damage that could be responsible for the unusual localization of PrP.

Although it seems unusual for a cell to have a protein in two such distinct locations (the membranes of the trafficking pathway and the cytosol), it is entirely plausible that a protein such as PrP\textsuperscript{Sc} could have roles in more than one compartment of a cell (Hegde and Lingappa, 1999). Such a diversity of function from a single gene has been observed for the protein calreticulin, which was found to function in the ER, cytosol, and nucleus (Smith and Koch, 1989; Burns et al., 1994; Coppolino et al., 1997). Similarly, dual localization of other proteins or protein domains has also been described for the plasminogen activator protein (Belin et al., 1996) and the hepatitis B virus envelope protein (Swameye and Schaller, 1997). It has been shown that PrP\textsuperscript{C} has a rather complex signal sequence, which directs it to the lumen of the ER (Zhang and Ling, 1995; Hegde et al., 1998; Holscher et al., 2001; Kim et al., 2001). Therefore, it is possible that the synthesis of the different topological forms of PrP\textsuperscript{C} varies between different cell types.

### Table 2. Quantification of PrP\textsuperscript{C} labeling on the membrane in different neuronal populations in the CA1 area and dentate gyrus\textsuperscript{a}

<table>
<thead>
<tr>
<th>Neurons</th>
<th>Endoplasmic reticulum</th>
<th>Golgi complex</th>
<th>Endosomes/lysosomes</th>
<th>Plasma membrane</th>
<th>Mitochondria</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyramidal (n = 18)</td>
<td>0.44 ± 0.03</td>
<td>0.71 ± 0.03</td>
<td>1.4 ± 0.2</td>
<td>1.37 ± 0.11</td>
<td>0.037 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>Hilal (n = 18)</td>
<td>0.23 ± 0.01</td>
<td>0.4 ± 0.01</td>
<td>0.59 ± 0.06</td>
<td>0.58 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Granule (n = 21)</td>
<td>0.25 ± 0.01</td>
<td>0.36 ± 0.03</td>
<td>0.58 ± 0.07</td>
<td>0.64 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>CPrP cells (n = 20)</td>
<td>0.38 ± 0.02</td>
<td>0.64 ± 0.05</td>
<td>1.51 ± 0.12</td>
<td>1.33 ± 0.08</td>
<td>0.06 ± 0.008</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are number of gold particles per 1 μm of membrane (gold per micrometer) in 77 cells from three different animals, given as mean ± SEM. Mitochondrial membranes were used to assess background labeling.

### Table 3. Quantification of gold-labeled PrP in the cytosol in different populations of neurons\textsuperscript{b}

<table>
<thead>
<tr>
<th>Neurons</th>
<th>Nucleus</th>
<th>Mitochondria</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyramidal (n = 18)</td>
<td>0.12 ± 0.04</td>
<td>0.87 ± 0.6</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Hilal (n = 18)</td>
<td>0.14 ± 0.08</td>
<td>1.09 ± 0.73</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Granule (n = 21)</td>
<td>0.11 ± 0.04</td>
<td>0.54 ± 0.26</td>
<td>0</td>
</tr>
<tr>
<td>CPrP cells (n = 20)</td>
<td>0.845 ± 0.16</td>
<td>2.6 ± 0.53</td>
<td>23.4 ± 3.6</td>
</tr>
</tbody>
</table>

\textsuperscript{b}Gold particles were quantified over an area of the cytosol that was free of organelles. To assess background labeling, gold particles were quantified over the area of mitochondria and nucleus (gold per square micrometer). Results are presented as mean ± SEM.

### Table 4. Observed and expected distributions of gold particles in compartments of pyramidal neurons and CPrP cells, relative labeling index, and \( \chi^2 \) values\textsuperscript{c}

<table>
<thead>
<tr>
<th>Compartments</th>
<th>Number of observed gold particles, ( n_o )</th>
<th>Number of grid points, ( P )</th>
<th>Normalized number of expected gold particles, ( n_e = P(n_o/total \ P) )</th>
<th>Relative labeling index, ( n_e/n_o )</th>
<th>Partial ( \chi^2 ) values, ( (n_o - n_e)^2/n_e )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyramidal neurons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>11</td>
<td>421</td>
<td>181</td>
<td>0.06</td>
<td>160</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>5</td>
<td>69</td>
<td>30</td>
<td>0.24</td>
<td>20</td>
</tr>
<tr>
<td>Cytosol</td>
<td>13</td>
<td>799</td>
<td>344</td>
<td>0.04</td>
<td>318</td>
</tr>
<tr>
<td>CPrP cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>26</td>
<td>227</td>
<td>98</td>
<td>0.27</td>
<td>51</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>27</td>
<td>105</td>
<td>45</td>
<td>0.6</td>
<td>7.2</td>
</tr>
<tr>
<td>Cytosol</td>
<td>908</td>
<td>678</td>
<td>292</td>
<td>3.12\textsuperscript{b}</td>
<td>1308\textsuperscript{b}</td>
</tr>
<tr>
<td>Totals</td>
<td>990</td>
<td>2299</td>
<td>990</td>
<td>1</td>
<td>1864.2\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{c}The values were quantified for 10 cells in each category.

\textsuperscript{b}Indicates compartment is preferentially labeled.

\textsuperscript{c}The distribution is nonrandom, with \( p < 0.001 \).
according to the expression of different cytoplasmic components of the translocation machinery (Hegde and Lingappa, 1999). The dislocation of PrP from the ER to the cytosol has been demonstrated previously in cell culture systems under certain conditions, such as in a reducing environment and glycosylation (Ma and Lindquist, 2001) or proteosomes (Yeddia et al., 2001) inhibition. This could explain the presence of cytPrP in situ. However, the absence of GPI-anchored Thy-1 in the cytosol of CprP cells argues that other GPI-anchored proteins preserve their usual localization. Because proteosome inhibition should affect many proteins, an unknown specific mechanism for PrP accumulation in the cytosol may be present.

It was recently shown that transfected cytPrP appears to be toxic in both cell culture and transgenic animals, in a cell type-dependent manner. Only cerebellar cells appeared to be affected in mice that expressed PrP C- without an ER translocation signal (Ma et al., 2002). Accumulation of cytPrP in “susceptible” neurons might be responsible for some of the variants of prion diseases in which cytPrP aggregates kill the cell and cause release of infectious prions. These prions might then initiate the vicious circle of prion propagation. However, our studies show that cytPrP is present in normal rodent brains in a population of neurons that appears healthy and shows no cellular degeneration. Thus, we can infer that cytPrP is not toxic in some neurons but highly toxic when overexpressed in specific cell populations. More work is needed to elucidate the causes of such striking differences resulting from cytPrP.

References


