Cellular prion protein co-localizes with nAChR β4 subunit in brain and gastrointestinal tract

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Abstract

PrP\textsuperscript{C}, the cellular isoform of prion protein, is widely expressed in most tissues, including brain, muscle and gastrointestinal tract. Despite its involvement in several bioprocesses, PrP has still no apparent physiological role. During propagation of transmissible spongiform encephalopathies (TSE), prion protein is converted to the pathological isoform PrP\textsuperscript{Sc}, in a process believed to be mediated by unknown host factors. The identification of proteins associated with PrP may provide information about both the biology of prions and the pathogenesis of TSE. Thus far, PrP\textsuperscript{C} has been shown to interact with synaptic proteins, components of the cytoskeleton and intracellular proteins involved in signalling pathways. Here, we describe the association of PrP with the ß4 subunit of nicotinic acetylcholine receptor (nAChR), as indicated by co-immunoprecipitation assays and double-label immunofluorescence. The interaction between prion protein and native ß4 subunit was further studied by affinity chromatography, using immobilized and refolded recombinant PrP as a bait and brain homogenates from normal individuals. Additionally, the participation of ß4 subunit in the pathogenesis of TSE was studied by in vivo assays. ß4\textsuperscript{−/−} and wild-type mice were challenged with the RML (Rocky Mountain Laboratories) infectious agent. Transgenic animals displayed altered incubation times but the deletion of ß4 subunit did not result in a significant change of the incubation period of the disease. Our results suggest that PrP\textsuperscript{C} is a member of a multiprotein membrane complex participating in the formation and function of ß3ß4 nAChR.

Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases caused by the accumulation of a pathological isoform of prion protein (PrP) (Prusiner, 1998). PrP\textsuperscript{C}, the cellular prion protein, is attached to the outer cell membrane via a glycosylphosphatidylinositol (GPI) anchor and is expressed in most tissues (Horiuchi et al., 1995; Moser et al., 1995; Pamminger et al., 2000). Its physiological role has not yet been determined. It has been proposed that PrP\textsuperscript{C} participates in the metabolism of copper (Pauly & Harris, 1998), transduces neuroprotective signals (Chiariini et al., 2002) or activates endogenous kinases (Mouillet-Richard et al., 2000).

A key event in prion diseases is the conversion to the pathological PrP\textsuperscript{Sc} isoform, which represents the major component of the infectious agent. The formation of PrP\textsuperscript{Sc} is accompanied by profound changes in the secondary structure and biochemical properties of prion protein. PrP\textsuperscript{C}, which mainly consists of Ï±-helices, converts to a Ï±-sheet-rich structure, which is partially resistant to proteolysis and has the capacity to form extracellular aggregates (Prusiner, 1998). In some cases, clinical symptoms may occur even without these characteristic PrP\textsuperscript{Sc} deposits (Collinge et al., 1990), suggesting that the aetiology of prion diseases might be the loss of PrP\textsuperscript{C} (Aguzzi & Weissmann, 1997).

The conversion mechanism remains undefined, but it is proposed to be mediated by as yet unknown host-encoded factors. The term protein X was introduced to describe such an undefined and species-specific macromolecule that facilitates the formation of PrP\textsuperscript{Sc} (Telling et al., 1995). Acting as a molecular chaperone, protein X is suggested to interact with PrP\textsuperscript{C} through a discontinuous epitope at the C-terminal part of PrP (Kaneko et al., 1997).

The identification of proteins interacting with prion protein has been the aim of many studies, owing to the importance of understanding both the physiological role of PrP\textsuperscript{C} and the conversion mechanism to the pathological PrP\textsuperscript{Sc} isoform. To date, prion protein has been described to interact with several molecules. These include extracellular matrix proteins and their receptors (Granet et al., 2000; Hundt et al., 2001), neural cell adhesion molecules (Schmitt-Ulms et al., 2001), structural protein complexes of the cell membrane and the cytoskeleton (Keshet et al., 2000; Petrakis & Sklaviadis, 2006), and intracellular proteins involved in signal transduction (Spielhaupter & Schatzl, 2001; Satoh et al., 2005).
In this study we describe the co-localization of PrP\(^{C}\) and \(\beta_4\) subunit of nicotinic acetylcholine receptor (nAChR) in brain tissue and gastrointestinal tract, and also the possible participation of this subunit in TSE pathogenesis. Our findings are in agreement with previous publications, showing that prion protein co-localizes with nAChRs in the neuromuscular junction (Askanas et al., 1998) and interacts with nAChR-associated proteins (Keshet et al., 2000; Spielhaupter & Schatzl, 2001; Petrikas & Sklavdiadis, 2006). These results suggest that PrP may play an important role in signal transduction in postsynaptic membranes.

Materials and methods

Tissues and antibodies

Human cerebral and ovine cerebral cortex from healthy individuals were kindly provided by Professor Plaitakis (University of Crete Medical School) and Dr P. Tournazos (Veterinary Services Laboratory, Cyprus), respectively. Autopsy material from human large bowel was provided by Dr T. Katsinelos (Gastrointestinal Unit, Genimatas General Hospital, Thessaloniki). All EU and Hellenic Republic regulations as well as the regulations of AUTH and INA/CERTH for use of human and animal tissues have been followed. Anti-PrP monoclonal antibody (mAb) 4F2 (Kraemann et al., 1996) was kindly provided by Professor Bodemer. Anti-PrP mAb 6H4 and anti-PrP pAb 915-011 were obtained from Prionics AG (Zurich, Switzerland) and Assay Designs (Michigan, MI, USA), respectively.

Antibodies against nAChR subunits were: anti-\(\beta_4\) mAb 337, raised against a bacterially expressed peptide (amino acids 305–419) from the large cytoplasmic domain of human \(\beta_4\) subunit (Nelson et al., 2001), anti-\(\alpha_3\) mAb 210, which recognizes the main immunogenic region of \(\alpha_1\), \(\alpha_3\) and \(\alpha_5\) subunits (Tzartos et al., 1987) and rat antiseraum against human \(\alpha_3\) subunit, adsorbed with recombinant human \(\alpha_5\) and \(\alpha_6\) subunits (produced in Professor Lindstrom’s laboratory). Anti-actin mAb conjugated to horseradish peroxidase (HRP) was obtained from Santa Cruz (Heidelberg, Germany). Secondary rabbit anti-mouse and goat anti-rat alkaline phosphatase-conjugated antibodies were purchased from Pierce (Rockford, IL, USA) and Santa Cruz, respectively. Secondary goat anti-mouse Rhodamine Red X (RRX) and goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibodies were obtained from Jackson (Suffolk, UK).

Preparation of the homogenates

Brain tissues were homogenized in nine volumes of 1% IGEPAL CA-630 phosphate-buffered saline (PBS), containing complete protease inhibitor cocktail mix (Sigma, St Louis, MO, USA), with a mortar and a pestle. The homogenates were centrifuged at 20 000g for 10 min and the supernatant was aliquoted and kept at \(-70^\circ\)C.

Co-immunoprecipitation assays

One microgram of anti-PrP mAb 4F2, 10 \(\mu\)g anti-\(\beta_4\) nAChR mAb 337 and 10 \(\mu\)g anti-\(\alpha_3\) nAChR mAb 210 were individually incubated with 50 \(\mu\)L of protein G-agarose beads (Upstate, Charlottesville, VA, USA) overnight at \(\pm 4^\circ\)C. Agarose beads were collected by centrifugation at 500g for 2 min and the antibody/protein G complex was covalently cross-linked with 4 mM DSP cross-linker (Pierce) for 1 h at \(\pm 4^\circ\)C. The remaining uncoupled cross-linker was blocked with 50 mM Tris-Cl, pH 7.5, for 1 h at \(\pm 4^\circ\)C. The beads were pre-eluted twice with 1 mL of 0.1 M glycine, pH 2.3, to remove non-covalently linked immuno-globulins. After centrifugation at 500g for 2 min, pH was neutralized with 1 mL of 1 M Tris-Cl, pH 7.5. The antibody/protein G-agarose beads were then mixed with 10 mg brain equivalent of the human brain homogenate. After an overnight incubation at \(\pm 4^\circ\)C, the beads were collected by centrifugation at 500g for 2 min and washed three times in 1% v/v IGEPAL CA-630 PBS. Bound proteins were eluted by boiling for 5 min in 30 \(\mu\)L O’Farrell sample buffer (O’Farrell, 1975), lacking the reducing agent. After centrifugation, the supernatant was transferred in new tubes, mixed with 5% v/v \(\beta\)-mercaptoethanol and analysed in a 12% SDS-PAGE.

On-column refolding of ovine recombinant PrP

Full-length ovine PrP (amino acids 23–234), with the addition of a His-tag at the N-terminus, was expressed in Escherichia coli BL21/DE3. Prion protein was immobilized on Ni-NTA agarose resin (Qiagen, Hilden, Germany) and on-column refolded. In brief, 40 mg of purified inclusion bodies, containing aggregated ovine recombinant PrP, was resuspended in 17 mL of Buffer A (6 mM guanidine HCl, 10 mM Tris-Cl, pH 8, 100 mM Na\(_2\)HPO\(_4\), 10 mM reduced glutathione) with stirring for 1 h at room temperature. The mixture was centrifuged at 20 000g for 15 min and the supernatant was incubated with 1 mL of Ni-NTA agarose beads, overnight at \(\pm 4^\circ\)C. The resin was then packed on a mini column, washed with 20 mL of Buffer A, followed by the application of a 35 mL linear gradient of Buffer A and Buffer B (10 mM Tris-Cl, pH 8, 100 mM Na\(_2\)HPO\(_4\)). The column with immobilized refolded PrP was washed with 12.5 mL of Buffer C (10 mM Tris-Cl, pH 8, 100 mM Na\(_2\)HPO\(_4\), 20 mM imidazole), pre-eluted with 7 mL of 0.1 M diethanolamine, pH 11, to remove impurities and stored at \(\pm 4^\circ\)C in PBS.

Affinity chromatography

PrP affinity chromatography was performed as previously described (Petrikas & Sklavdiadis, 2006). In brief, 1 mL of a 10% v/v human or ovine brain homogenate was diluted two-fold separately with lysis buffer [PBS containing 0.5% v/v Igepal CA-630 and 0.5% sodium deoxycholate (DOC)] and incubated for 10 min at room temperature. Mixtures were centrifuged at 20 000g for 10 min and supernatants were further diluted with PBS to a final volume of 10 mL. Homogenates were incubated with 1 mL of Ni-NTA beads overnight at \(\pm 4^\circ\)C in order to remove proteins with affinity for the resin. The suspensions were packed on a mini-column and precleared fractions were applied on columns with immobilized refolded recombinant ovine prion protein and remained overnight at \(\pm 4^\circ\)C. Columns were washed with 100 mL of wash buffer I (0.05% w/v DOC, 0.15% v/v Igepal CA-630 PBS), 20 mL of wash buffer II (0.125% w/v DOC, 0.375% v/v Igepal CA-630 PBS) and 20 mL of wash buffer III (0.25% w/v DOC, 0.75% v/v Igepal CA-630 PBS). Finally, PrP-associated proteins were eluted with 0.5 mL of 0.1 M diethanolamine, pH 11, and pH was neutralized with 0.25 mL 0.3 M Tris-Cl, pH 8. Chromatography fractions were methanol-precipitated with nine volumes of ice-cold methanol overnight at \(\pm 70^\circ\)C and centrifuged at 20 000g for 10 min. The resulting pellets were resuspended in 30 \(\mu\)L O’Farrell sample buffer, boiled for 5 min and analysed in a 12% SDS-PAGE.

Western blotting

After electrophoresis, proteins were electrotransferred onto nitrocellulose membrane (NT) (Pall Life Sciences, East Hills, NY, USA) for 2 h at 100 V. Membranes were blocked with 5% w/v bovine serum albumin (BSA), 0.1% v/v Tween 20 PBS (PBST) for 1 h at room temperature and then incubated with primary antibody at an appropriate dilution for 1 h at room temperature. Membranes were washed three times with 1% v/v Igepal CA-630 PBS, 20 mL of wash buffer II (0.125% w/v DOC, 0.375% v/v Igepal CA-630 PBS) and 20 mL of wash buffer III (0.25% w/v DOC, 0.75% v/v Igepal CA-630 PBS). Finally, PrP-associated proteins were eluted with 0.5 mL of 0.1 M diethanolamine, pH 11, and pH was neutralized with 0.25 mL 0.3 M Tris-Cl, pH 8. Chromatography fractions were methanol-precipitated with nine volumes of ice-cold methanol overnight at \(\pm 70^\circ\)C and centrifuged at 20 000g for 10 min. The resulting pellets were resuspended in 30 \(\mu\)L O’Farrell sample buffer, boiled for 5 min and analysed in a 12% SDS-PAGE.

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temperature, followed by incubation with the primary antibodies. The antigens were detected with anti-β4 nAChR mAb 337 (1 : 1000), α3 antisera (1 : 500), anti-PrP mAb 6H4 (1 : 5000) or anti-actin HRP-conjugated mAb (1 : 500), overnight at 4 °C. The membranes were washed 1 × 15 min and 3 × 5 min in PBST and were then incubated with the appropriate secondary alkaline-phosphatase-conjugated antibody (1 : 5000). After washes in PBST, the blots were developed with NBT/BCIP or ECL (Sigma), according to the manufacturer’s instructions.

Double-label immunofluorescence
Normal C57/Bl6 mice were anaesthetized and transcardially perfused with PBS, followed by 4% v/v paraformaldehyde (PFA) PBS. The brain was removed and fixed in 4% v/v PFA PBS for 72 h. Human tissue from large bowel was similarly fixed in 4% v/v PFA PBS. All tissues were then processed for embedding in paraffin. Five-micrometre-thick formalin-fixed paraffin-embedded sections were prepared, rehydrated and treated with 10 mM citrate buffer, pH 6, for 10 min in a microwave oven (250 W). The sections were blocked with 5% w/v BSA, 10% v/v normal goat serum (NGS) PBS for 1 h at room temperature, followed by incubation with anti-β4 nAChR mAb 337 (1 : 100 in 2.5% w/v BSA, 5% v/v NGS PBS), overnight at 4 °C. The slides were washed 4 × 10 min in PBS and incubated with the secondary goat anti-mouse RRX antibody (1 : 50 in 2.5% w/v BSA, 5% v/v NGS PBS) for 2 h at room temperature. After 4 × 10-min washes in PBS, the sections were blocked again with 5% w/v BSA, 10% v/v NGS PBS for 1 h at room temperature and then probed with anti-PrP pAb 915-011 (1 : 50 in 2.5% w/v BSA, 5% v/v NGS PBS), overnight at 4 °C. The sections were washed 4 × 10 min in PBS and incubated with the secondary goat anti-rabbit FITC-conjugated antibody (1 : 50 in 2.5% w/v BSA, 5% v/v NGS PBS) for 2 h at room temperature. All incubations were performed in humidity chambers. Finally, the slides were washed 4 × 10 min in PBS, covered with mounting medium and observed with a confocal microscope.

Confocal laser scanning microscopy
Confocal laser scanning microscopy was performed using an SP2 confocal microscope (LEICA Microsystems, Mannheim, Germany) mounted on an inverted microscope. Images were acquired with a 63× objective (NA = 1.32). Acquisition was with the Ar laser (excitation 488 nm, emission 500–540 nm) for FITC and the HeNe laser (excitation 543 nm, emission 633 nm) for RRX in a sequential way to avoid cross-talk. Co-localization studies were performed using the LEICA software, based on the cytofluorogram. A cytofluorogram is a dot diagram that visualizes the joint distribution of intensity values of the green and red channels. Every dot of the scatter plot of the cytofluorogram represents an intensity value pair from the two detection channels. Points in white represent the co-localization regions, defined as the dots inside the rectangular region of the cytofluorogram.

Bioassays
Groups of adult β4−/− (n = 5) and wild-type (n = 9) C57/Bl6 mice of either sex were inoculated intraperitoneally with 100 µL of a 1% w/v brain homogenate in PBS, originating from mice infected with the RML strain. All procedures were reviewed and approved by the ethics committee of the Hebrew University Medical School. Mice were then monitored for the progression of disease. All animals were killed at the terminal stages of the disease by brief anaesthesia using ether and then cervical dislocation. The presence of PrPSc in brain tissue from both β4−/− and wild-type C57/Bl6 mice was verified using a short purification protocol, as previously described (Poliomenidou et al., 2002). Survival curves were drawn using GraphPad Prism software.

Results
PrPSc and nAChR β4 subunit co-immunoprecipitate in normal brain tissue
In this study we describe the association between PrPSc and nAChR β4 subunit. This subunit is specific for nicotinic receptors and participates in the formation of nAChR ion channels in the central and peripheral nervous system. In previously performed phage display experiments with recombinant human PrP as target, we identified β4 subunit as a candidate PrP interacting protein (manuscript in preparation). Prompted by these results we carried out co-immunoprecipitation assays using antibodies against prion protein and nAChR α3, β4 subunits. Normal human brain extracts were immunoprecipitated with either anti-PrP mAb 4F2 or anti-β4 mAb 337 and immunoblotted for the presence of β4 subunit and PrPSc. When probing PrP immunoprecipitations with anti-β4 mAb 337, we consistently detected a dominant band co-purifying with prion protein at approximately 55 kDa. The same band was also detected in β4 immunoprecipitations, which served as a positive control for this subunit (Fig. 1a). Conversely, PrPSc was detected in β4 subunit immunoprecipitations as well, suggesting that both proteins localize in the same microdomains of the cell membrane (Fig. 1b). In all cases, control experiments were performed in the absence of either brain extracts or immunoglobulins.

PrPSc was also detected in nAChR α3 subunit immunoprecipitations, which along with its partner β4 form the α3β4 receptor (Fig. 1c). The
increased susceptibility to autolysis (Martin-Ruiz et al. 1997). The latter observation would be the proteolysis of proteins originating from human cerebellum and ovine cerebral cortex, were probed using mass spectroscopy or Western blotting (Petrakis & Sklaviadis, 2006). Columns were washed with buffers containing increasing concentrations of detergents to enhance the stringency of the experimental conditions. Several proteins were isolated and identified using mass spectrometry. Normal ovine and human brain homogenates were prepared and passed over PrP columns. Bound proteins were eluted and affinity chromatography fractions were analysed via SDS-PAGE and transferred onto NT membranes. Proteins originating from human cerebellum (Lane 1) or ovine cerebral cortex (Lane 2) were probed with either anti-β4 nAChR mAb 337, to detect β4 subunit (Lanes 1 and 2, upper panel) or α3 rat antisera, to detect α3 subunit (Lanes 1 and 2, lower panel).

Reduced amount of prion protein detected in α3 subunit co-immunoprecipitation assay may be due to the fact that mAb 210 recognizes not only α3, but to some degree α1 and α5 subunits as well. This observation suggests that PrPC is possibly associated only with a specific subpopulation of nicotinic receptors.

**NACHR α3 and β4 subunits bind to refolded ovine recombinant PrP**

The interaction between PrP and α3β4 nACHR was further studied by in vitro binding assays. Proteins originating from normal brain tissue were affinity purified based on their ability to bind on immobilized prion protein. Normal ovine and human brain homogenates were prepared and chromatographed over refolded PrP affinity columns. Columns were washed with buffers containing increasing concentrations of detergents to enhance the stringency of the experimental conditions. Several proteins were isolated and identified using mass spectroscopy or Western blotting (Petrakis & Sklaviadis, 2006). Affinity chromatography fractions, which contained proteins originating from human cerebellum and ovine cerebral cortex, were probed for the presence of nACHR α3 and β4 subunits. Using anti-β4 mAb 337 and α3 rat antisera, we detected both subunits at approximately 55 and 36 kDa, respectively (Fig. 2). β4 subunit was detected at the correct molecular weight, while α3 subunit was probed with a much lower molecular mass than expected. A possible explanation for the latter observation would be the proteolysis of α3 subunit, as reported by Martin-Ruiz et al. (2000). They also described the detection of α3 subunit in human frontal cortex at a lower molecular weight. The difference between the expected (59 kDa) and the observed molecular weight (44 kDa) may reflect the possibility that the cellular compartment where α3 subunit is located demonstrates increased susceptibility to autolysis (Martin-Ruiz et al., 2000). Taken together, the previous results suggest that α3β4 nACHR either interacts directly with PrP or is a part of a protein complex which is associated with prion protein.

**Co-localization of PrPC and nACHR β4 subunit**

Preliminary results from serial sections stained for either PrPC or β4 subunit showed that both proteins are expressed in the same regions of the brain (data not shown). To verify the co-localization of prion protein and nACHR β4 subunit, we performed double-label immunofluorescence in normal murine brain tissue, using anti-PrP pAb 915-011 and anti-β4 mAb 337. Control experiments were performed in the absence of primary antibodies or using scrambled combinations of primary and secondary antibodies. Additionally, the specificity of mAb 337 for β4 subunit was tested in brain tissue sections from β4 knockout animals. In all cases, these experiments were negative (data not shown). Double-label immunofluorescence and confocal microscopy showed that PrPC and nACHR β4 subunit are both expressed in cerebral cortex and hippocampus of murine brain, co-localizing in the periphery of the neuronal cells. Co-localization regions in the cerebral cortex are shown by either a merged green–red colour (Fig. 3a, C) or by white spots that indicate the joint distribution of two colours (Fig. 3a, D). Differential interference contrast (DIC) images were used to represent the cells of the neuronal tissue (Fig. 3a, F).

α3β4 is the main nACHR receptor of the peripheral nervous system (Lindstrom et al., 1996). Immunohistochemical studies have already shown the presence of nACHR α3, β4 subunits and PrPC in the gastrointestinal tract (Pammer et al., 2000; Obaid et al., 2005) to investigate the possibility that PrPC and nACHR β4 subunit are also associated in the periphery, similar experiments were performed in sections from human large bowel. Prion protein was detected in the epithelial cells of the mucosa and the lymphocytes present in lamina propria (Fig. 3b, A). Immunoactivity for β4 subunit was also observed in peripheral epithelium cells (Fig. 3b, B), suggesting that both proteins co-localize in gastrointestinal tract (Fig. 3b, C and D).

**Bioassays**

The gastrointestinal tract and especially the M cells of the epithelium represent the main entry point of prions in the organism (Heppner et al., 2001). The co-localization of nACHR β4 subunit and PrPC in the large bowel suggests that this subunit may participate in the uptake of the infectious agent and results in peripheral nervous system pathogenesis. To investigate this hypothesis, β4−/− nACHR and wild-type C57/B16 mice were challenged with mouse prions (RML strain). Both groups of animals express similar levels of PrPC in brain (Fig. 4a). After i.p. inoculation, all mice displayed the classical symptoms of prion disease. The animals were killed at terminal stages of the disease and the presence of PrPsc was verified in brain tissue from both groups of animals (Fig. 4b). Interestingly, β4−/− nACHR mice displayed slightly extended incubation times, after i.p. inoculation with prions (Fig. 4c). The mean incubation time for wild-type mice was 224 days post infection (dpi), while knockout animals had a mean incubation time of 228 dpi. Even though these differences are not statistically significant, they could possibly mean that nACHR β4 subunit may participate in one of the uptake routes of the infectious agent in early stages of pathogenesis.

**Discussion**

In this study we described the co-localization of prion protein and nACHR β4 subunit in normal brain tissue and gastrointestinal tract. Performing co-immunoprecipitation assays with anti-PrP mAb 4F2, we show that the nACHR β4 subunit which co-precipitated with PrPC prion protein is also detected when performing co-immunoprecipitation assays with antibodies against α and β subunits of the α3β4 nACHR. Association between PrPC and nACHR β4 subunit was further shown by double-label immunofluorescence and in vitro binding assays, using affinity chromatography techniques.
Fig. 3. (a) Co-localization of PrP<sup>C</sup> and β4 nAChR subunit in brain. Five-micrometre-thick sections from formalin-fixed paraffin-embedded murine brain were prepared. Double-label immunofluorescence was performed using anti-PrP pAb 915-011/FITC-conjugated secondary antibody and anti-β4 subunit mAb337/RRX-conjugated secondary antibody. Images represent single confocal sections. (A) PrP<sup>C</sup> staining, (B) β4 nAChR staining in cerebral cortex, (C) merged picture, (D) co-localization regions shown with white spots, (E) cytofluorogram, showing the joint distribution of green and red colours. The rectangular scheme defines the co-localization regions. (F) Differential interference contrast images (DIC), displaying the cells. (b) Co-localization of PrP<sup>C</sup> and β4 nAChR subunit in gastrointestinal tract. Five-micrometre-thick sections from formalin-fixed paraffin-embedded human large bowel were prepared. Double-label immunofluorescence was performed using anti-PrP pAb 915-011/FITC-conjugated secondary antibody and anti-β4 subunit mAb337/RRX-conjugated secondary antibody. Images represent single confocal sections (A) PrP<sup>C</sup> staining in epithelial cells and lymphocytes, (B) β4 nAChR staining in epithelial cells, (C) merged picture, (D) co-localization regions, shown with white spots, (E) cytofluorogram, showing the joint distribution of green and red colours. The rectangular scheme defines the co-localization regions. (F) Differential interference contrast images (DIC), displaying the cells.
nAChRs belong to a gene superfamily of homologous receptors including glycine, \( \gamma \)-aminobutyric acid (GABA) and serotonin receptors and are widely expressed in muscle, nervous system and non-neuronal tissues. Muscle nicotinic receptors are composed of a fixed subunit composition \([\alpha_1]_2\beta_1\epsilon_d\] in adult organisms and their role in postsynaptic regions and neuromuscular junctions is well known. On the other hand, neuronal receptors display a great variation in subunit composition and are found in both pre- and postsynaptic locations. They can participate directly in neurotransmission or modulate neurotransmitter release and affect neuronal growth during development and regeneration (Lindstrom et al., 1996).

Neuronal nAChRs can be subdivided in two distinct subclasses; the first includes homopentameric receptors formed by \( \alpha_7 \), \( \alpha_8 \) or \( \alpha_9 \) subunits. The second one contains heteropentameric receptors that can form functional nAChRs only when \( \alpha_2 \), \( \alpha_3 \), \( \alpha_4 \) or \( \alpha_6 \) subunits are combined with either \( \beta_2 \) or \( \beta_4 \) subunits. \( \beta_4 \) along with its main partner, \( \alpha_3 \) subunit, form the \( \alpha_3 \beta_4 \) nAChR, which is expressed in the central and peripheral nervous system and autonomic ganglia (Lindstrom et al., 1996). Recent studies show a wide distribution of \( \beta_4 \) subunit in the adult mouse brain, including sensory systems and the hippocampus (Gahring et al., 2004) and also indicate that \( \alpha_3 \beta_4 \) is the main nAChR in several brain structures, such as the dorsal horn of the spinal cord, the superior cervical ganglion and adrenal glands (Perry et al., 2002).

The first evidence that prion protein might be associated with acetylcholine receptors was provided when a chicken prion-like protein (ch-PrLP) co-purified with an acetylcholine receptor-inducing activity (ARIA) chromatography fraction. ARIA, isolated from chicken brain, could stimulate the synthesis of acetylcholine receptors in cultured chicken myotubes and PrLP was the only protein identified in this fraction. Immunohistochemistry showed that ch-PrLP is concentrated in cholinergic neurons and its mRNA

![Fig. 4](image-url)

**Fig. 4.** (a) PrP\( ^C \) expression levels in brain of \( \beta_4^– \) and wild-type mice. Brain homogenates (2 mg b.e.) from \( \beta_4 \) knockout and wild-type mice were analysed via SDS-PAGE, transferred onto NT membranes and probed for PrP\( ^C \) or \( \beta \)-actin, with anti-PrP mAb 6H4 or HRP-conjugated anti-\( \beta \)-actin mAb, respectively. (b) PrP\( ^C \) detection in brain of \( \beta_4^– \) and wild-type mice. Brain homogenates (5 mg b.e.), from either \( \beta_4^– \) (Lanes 1–3) or wild-type mice (Lanes 4–6), were enriched in PrP\( ^\text{Sc} \), after PK treatment. Both preparations were analysed via SDS-PAGE, transferred onto NT membranes and probed with anti-PrP mAb 6H4. (c) Survival curves of \( \beta_4^– \) and wild-type mice after i.p. inoculation with Mo prions (RML strain). Hypothetical model of signal transduction mediated by PrP\( ^C \). (a) PrP\( ^C \) along with the DGC, agrin, laminin and membrane proteins (CNPase, BASP1) cluster \( \alpha_3\beta_4 \) nAChRs in the cell membrane and link them to the cytoskeleton. PrP\( ^C \) activates tyrosine kinases (e.g. CK2) that phosphorylate \( \beta \)-DG and nAChR subunits. (b) The interaction between \( \beta \)-DG and Dp is disrupted and Grb2 forms a stable complex with \( \beta \)-DG and \( \alpha_3\beta_4 \) nAChR. Grb2 and its partner, synapsin I, control vesicle circulation and regulate signal transduction in synapses. Other molecules, such as STXBP1 and PLP, may contribute to the function of the receptor.
levels increase in parallel with choline acetyltransferase activity in the basal forebrain (Harris et al., 1991). Prion protein co-localizes with nicotinic receptors in the neuromuscular junction (NMJ). PrP is located in the postsynaptic membranes of human NMJ along with nAChRs, β-amyloid precursor protein (β-APP) and dystrophin (Askanas et al., 1998). For the first time we provide evidence that PrP co-localizes with nAChR β4 subunit in brain and gastrointestinal tract.

Several proteins are associated with both PrP and nAChRs in brain, such as the dystroglycan complex (DGC), laminin and Grb2. The DGC links the outer membrane to the intracellular surface. It consists of α- and β-dystroglycan (α-DG and β-DG) and dystrophin (Dp). Dp is located in postsynaptic regions and has an actin-binding domain to the cytoskeleton. In brain, PrP interacts with β-DG, the transmembrane core of the DGC and Dp (Keshet et al., 2000). α-DG, the extracellular component of DGC, binds to agrin and the α chains of laminin (Blake & Kroger, 2000). The DGC complex, agrin and laminin are essential for the proper formation and clustering of nAChRs, as mice deficient in these molecules develop muscular dystrophy and have disrupted NMJs (Cote et al., 1999; Lee et al., 2002). These data suggest that the DGC plays a rather important role in nAChR formation and stabilization in the cell membrane.

Synapsin I and Grb2 have been identified as PrP-interacting proteins as well, in a yeast two-hybrid system (Spielhaupter & Schlitz, 2001). Synapsins play an important role in the formation of synapses and the release of neurotransmitters by regulating vesicle circulation. They interact with actin and cross-link vesicles to the cytoskeleton. Grb2 is an adaptor protein involved in signal transduction that links signals coming from extracellular or transmembrane receptors to intracellular molecules. It contains binding sites for PrP (Lysek & Wuthrich, 2000) and activates signalling pathways, interacting with tyrosine kinases. Grb2 interacts with nAChRs and β-DG, as well. In both cases the interaction is facilitated by tyrosine phosphorylation of nAChRs (Colledge & Froehner, 1997) and β-DG (Cavaldesi et al., 1999). Grb2 interacts with β-DG on an epitope that overlaps with the dystrophin-binding site, suggesting that Grb2 and Dp compete for binding to the DGC. Thus, there should be a regulatory mechanism allowing the interaction of either Grb2 or Dp with β-DG (Russo et al., 1999). In muscle the binding of utrophin, a dystrophin homologous protein, to the DGC is modulated by Ca²⁺/calmodulin and tyrosine phosphorylation of β-DG (James et al., 2000). Such regulatory mechanisms may also exist in neuronal tissue, allowing the binding of either Dp or Grb2 to the DGC.

Such a hypothesis would require the activation of intracellular phosphorylation systems. Prion protein has been described to activate a number of endogenous kinases involved in phosphorylation pathways. PrP fibrils activate tyrosine kinases in microglia cell cultures, through Ca²⁺/calmodulin and cAMP signalling pathways (Combs et al., 1999). Antibody-mediated cross-linking of PrPC to the cell membrane also activates Fyn, and Fyk tyrosine kinases in differentiated 1C11 neuronal cells (Mouillet-Richard et al., 2000).

We have recently described the characterization of several brain proteins with affinity for native or refolded recombinant PrPC, such as α-spectrin, CNPase, BASP1, STXBP1, PLP, Na⁺/K⁺ ATPase α3 subunit and CK 2 (Petrakis & Sklaviadis, 2006). Most are associated with ion channels. These proteins may regulate the formation and function of nAChRs, providing mechanical linkage to the cytoskeleton or activating intracellular signalling pathways.

Based on our results and the bibliographic data mentioned above, we propose that PrP along with the DGC, agrin and laminin form a skeleton which facilitates the formation and clustering of α3β4 nAChR in brain. The anchoring of the receptor to the cytoskeleton may be mediated by the DGC or transmembrane proteins, such as CNPase, BASP1 and syntaxins. Prion protein may also activate intracellular phosphorylation systems, such as CK2. PrP-induced kinases could phosphorylate nAChR subunits or β-DG. The phosphorylation of β-DG may result in the replacement of Dp with Grb2 in the DGC complex and the formation of a PrP–β-DG–Grb2–α3β4 nAChR complex. Grb2 could modulate the permeability and function of the ion channels and, along with its partner synapsin I, regulate vesicle trafficking. So, in conclusion, prion protein may either contribute to the stabilization of α3β4 nAChR on the cell membrane (Fig. 5a) or regulate signal transduction from pre- to postsynaptic membranes in the nervous system (Fig. 5b). This hypothesis is also strengthened by observations in PrP−/− mice that display weakened GABA_A receptor activity, impaired long-term potentiation and disruption of K⁺ currents. Taken together, all these data suggest that prion protein is necessary for normal synaptic function. Further electrophysiological experiments or tests with nAChR-specific chemical

![Fig. 5. Hypothetical model of signal transduction mediated by PrP]. (a) PrP along with the DGC, agrin, laminin and membrane proteins (CNPase, BASP1) cluster α3β4 nAChRs in the cell membrane and link them to the cytoskeleton. PrP activates tyrosine kinases (e.g. CK2) that phosphorylate β-DG and nAChR subunits. (b) The interaction between β-DG and Dp is disrupted and Grb2 forms a stable complex with β-DG and α3β4 nAChR. Grb2 and its partner, synapsin I control vesicle circulation and regulate signal transduction in synapses. Other molecules, such as STXBP1 and PLP may contribute to the function of the receptor.
compounds would provide more information on the metabolism of PrP\(^\text{Sc}\) and the function of nicotinic receptors.

The participation of nAChR \(\beta4\) subunit in TSE pathogenesis was studied by in vivo experiments. Bioassays with \(\beta4^{-}\) mice showed that knockout animals displayed slightly extended incubation times of prion disease than wild-type mice. These differences are not statistically significant and indicate that nAChR \(\beta4\) subunit is not crucial for peripheral pathogenesis of prion disease. However, we should keep in mind that the absence of \(\beta4\) subunit may result in the activation of an alternative neuroinvasion pathway, as its function could be compensated for by another receptor with similar structure.

As mentioned before, the identification of proteins interacting with PrP has been the aim of many research groups. The inhibition of these interactions might lead to the development of novel therapeutic approaches against prion diseases. The malfunction of PrP\(^\text{Sc}\) and its amyloidogenic properties may result in neuronal death, which is a common feature in TSE. nAChRs have already been involved in other neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, in which their number is gradually reduced, in parallel with neuronal loss (Clementi et al., 2000). Similar mechanisms may also exist in prion diseases. The association between PrP\(^\text{Sc}\) and \(\alpha3\beta4\) nAChR, described here, may shed more light on both the physiological role of prion protein and TSE pathogenesis.

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Abbreviations

\(\alpha\)- and \(\beta\)-DG, \(\alpha\)- and \(\beta\)-dystroglycan; ARIA, acetylcholine receptor-inducing activity; BASP1, brain abundant signal protein 1; \(\beta\)-APP, \(\beta\)-amyloid precursor protein; ch-PrLP, chicken prion-like protein; CK2, creatine kinase 2; CNPase, myelin basic protein; nAChR, nicotinic acetylcholine receptor; CK2, creatine kinase 2; BASP1, brain abundant signal protein 1; CNPase, activity; BASP1, brain abundant signal protein 1; nAChR, described here, may shed more light on both the physiological role of prion protein and TSE pathogenesis.

References


