Disease-associated mutations in the prion protein impair laminin-induced process outgrowth and survival

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Running title: PrP\textsuperscript{C} mutations impair laminin-induced process outgrowth and survival

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Background: In addition to the toxicity mediated by prion protein misfolding, mechanisms associated with its loss-of-function in genetic prion diseases are unknown.

Results: Neural cells expressing PrP\textsuperscript{C} mutants associated with prion diseases present impaired laminin-mediated process outgrowth and survival.

Conclusion: PrP\textsuperscript{C} mutants show loss-of-function in neural cells.

Significance: The impairment of prion protein functions may contribute to the etiology of prion diseases.

SUMMARY

Prions, the agents of transmissible spongiform encephalopathies, require the expression of prion protein (PrP\textsuperscript{C}) to propagate disease. PrP\textsuperscript{C} is converted into an abnormal insoluble form, PrP\textsuperscript{Sc}, which gains neurotoxic activity. Conversely, clinical manifestations of prion disease may occur either before, or in the absence of PrP\textsuperscript{Sc} deposits but the loss of normal PrP\textsuperscript{C} functions contribution for the etiology of these diseases is still debatable. Prion disease-associated mutations in PrP\textsuperscript{C} represent one of the best models to understand the impact of PrP\textsuperscript{C} loss-of-function. PrP\textsuperscript{C} associates with various molecules, and in particular, the interaction of PrP\textsuperscript{C} with laminin modulates neuronal plasticity and memory formation. To assess the functional alterations associated with PrP\textsuperscript{C} mutations, wild-type and mutated PrP\textsuperscript{C} proteins were expressed in a neural cell line derived from a PrP\textsuperscript{C}-null mouse. Treatment with the laminin γ\textsubscript{1} chain peptide (Ln γ\textsubscript{1}), which mimics the laminin binding site for PrP\textsuperscript{C}, increased intracellular calcium in cells expressing wild-type PrP\textsuperscript{C}, while a significantly lower response was observed in cells expressing mutated PrP\textsuperscript{C} molecules. The Ln γ\textsubscript{1} did not promote process outgrowth or protected against staurosporine induced cell death in cells expressing mutated PrP\textsuperscript{C} molecules in contrast to cells expressing wild-type PrP\textsuperscript{C}. The co-expression of wild-type PrP\textsuperscript{C} with mutated PrP\textsuperscript{C} molecules was able to rescue the Ln protective effects indicating the lack of negative dominance of PrP\textsuperscript{C} mutated molecules. These results indicate that PrP\textsuperscript{C} mutations impair process outgrowth and survival mediated by Ln γ\textsubscript{1} peptide in neural cells, which may contribute to the pathogenesis of genetic prion diseases.

INTRODUCTION

The cellular prion protein (PrP\textsuperscript{C}) is a glycosylphosphatidylinositol (GPI)-anchored protein whose conformationally modified isoform, PrP\textsuperscript{Sc}, is the major component of prions. Prions may be defined as infectious agents that cause the neurodegenerative diseases known as transmissible spongiform encephalopathies
forms (1). The accumulation of the toxic, insoluble PrP\textsuperscript{Sc} has been described to be the most likely event responsible for neuronal death in prion diseases (2). This idea is supported by the findings that deletion of PrP\textsuperscript{C}, or its knockdown in early stages of the disease, reverted spongiosis, neuron loss, cognitive and behavioral deficits, as well as impaired neurophysiological function in mice (3; 4; 5).

On the other hand, neuronal loss may be observed even in the absence of characteristic PrP\textsuperscript{Sc} deposits in sporadic, genetic, and iatrogenic forms of prion diseases (6; 7; 8). Small, punctate synaptic deposits of PrP\textsuperscript{Sc} correlate with neuronal loss, while the larger, focal types of deposits show an inverse correlation with neuronal counts (9). These findings could be related to the generation of oligomers of low molecular weight, present in brains of sporadic Creutzfeldt-Jakob disease patients, that control the progression rate of the disease (10). Remarkable, neurotoxicity was associated not only with PrP\textsuperscript{C} interaction with PrP\textsuperscript{Sc} but also with other β-sheet-rich conformers of different origin in which toxicity was independent of prions replication (11). Whether toxic signaling is due to gain-of-function of PrP\textsuperscript{C} or by the impairment of a normal signaling is unknown. Indeed, it is possible that PrP\textsuperscript{C} loss-of-function may also play a major role in the etiology of these maladies (12; 13; 14).

Interestingly, PrP\textsuperscript{C}-null mice do not have an obvious phenotype, implying that PrP\textsuperscript{C} may not have an essential function, or that its loss may be compensated for other molecules (15; 16). However, the expression of mutated PrP\textsuperscript{C} proteins carrying various deletions result in diverse phenotypic abnormalities that parallel neurodegenerative processes. These findings are consistent with the existence of domains within the PrP\textsuperscript{C} protein that have physiologically-relevant functions (17). In fact, PrP\textsuperscript{C} domains have been shown to be involved with some neurotrophic functions (14). Remarkably, several disease-associated mutations in PrP\textsuperscript{C} have been localized to these domains and, although these mutations account for only a small fraction of prion diseases, mutated PrP\textsuperscript{C} proteins provide us with an ideal model system with which to study PrP\textsuperscript{C} loss-of-function.

The genetic human spongiform encephalopathies, including Creutzfeldt-Jakob disease (CJD), Fatal Familial Insomnia (FFI), and Gerstmann-Straussler-Scheinker disease (GSS), are among the TSEs that have been linked to specific mutations in the PRNP gene that encodes PrP\textsuperscript{C} (18; 19). A common human PrP\textsuperscript{C} polymorphism at amino acid residue 129, which may be either a methionine or a valine, specifies the disease phenotype associated with an aspartate to glutamine mutation at position 178 (D178N). Specifically, the 129M/178N haplotype is linked to FFI, whereas the 129V/178D haplotype causes CJD (20). These disorders are autosomal dominant, fully penetrant inherited conditions, and the age of disease onset is around 50 years (78).

In the last decade, a diverse range of functions has been attributed to the native PrP\textsuperscript{C} protein, such as neuroprotection against cellular and systemic insults, neuritogenesis, neuronal plasticity and excitability, and memory formation and consolidation (21; 22; 23; 24; 25; 26; 27; 28; 29; 30; 31; 32; 33). Noteworthy, PrP\textsuperscript{C} mutations associated to CJD, FFI and GSS decrease or abolish the anti-bax function in primary human neurons and breast cancer cell lines promoting programmed cell death (34).

A vast number of ligands and signaling pathways have also been associated with PrP\textsuperscript{C} functions (79), and PrP\textsuperscript{C} has been proposed to organize a dynamic cell surface platform for the assembly of signaling modules (13). In addition, the neurotrophic functions attributed to PrP\textsuperscript{C} may reside in its ability to drive the assembly of multi-component complexes at the cell surface (14). Laminin (Ln), an extracellular matrix protein, is one of the high affinity ligands for PrP\textsuperscript{C} (24). The mouse PrP\textsuperscript{C}-Ln binding sites map to amino acids (aa) 1575–1584 (RNIAEIIKDI) in the γ1 chain region of Ln (Ln γ1; 24), and to aa 173–182 in PrP\textsuperscript{C} (22). Administration of the Ln γ1 peptide, which represents the PrP\textsuperscript{C} binding site, induces
PrP<sup>C</sup>-dependent neuritogenesis and reproduces the neuronal maturation phenotype that is dependent upon the binding of Ln to PrP<sup>C</sup> (24). The PrP<sup>C</sup>-Ln γ<sub>1</sub> interaction requires the activity of the group I metabotropic glutamate receptors, mGluR1 and mGluR5, to promote neuritogenesis through activation of phospholipase C and intracellular Ca<sup>2+</sup> mobilization (35). The formation of a PrP<sup>C</sup>-Ln-mGluR1/5 signaling complex is consistent with the scaffold properties of PrP<sup>C</sup> and its possible role in allosteric regulation of signal transduction (36). In agreement with the idea that the PrP<sup>C</sup>-Ln γ<sub>1</sub> peptide interaction induces physiological signals in the nervous system, the activity of this complex has been shown to enhance memory consolidation (22).

In the present report, we determined whether PrP<sup>C</sup> mutations associated with genetic prion diseases corrupt PrP<sup>C</sup>-dependent signaling pathways. The wild-type mouse PrP<sup>C</sup>, as well as mouse PrP<sup>C</sup> carrying mutations at codons 101L, 104L, 116V, 177N, 179I, and 199K (equivalent to human mutated proteins 102L, 105L, 117V, 178N, 180I, and 200K, respectively) were expressed in cells immortalized from primary neural cultures derived from PrP<sup>C</sup>-null mice. The exogenous wild-type and mutant PrP<sup>C</sup> proteins were evaluated for cell membrane expression and proteinase K resistance. The ability to interact with the Ln-γ<sub>1</sub> chain peptide upon Ca<sup>2+</sup> signaling to effect process outgrowth and induction of protective response was also determined. Our findings contribute to the identification of cellular mechanisms associated with PrP<sup>C</sup> loss-of-function.

**EXPERIMENTAL PROCEDURES**

**Peptides and Chemicals**

The Ln γ<sub>1</sub> peptide (RNIAEIIKDI) linked to BSA and Ln γ<sub>1</sub> scrambled (SCR) peptide (IRADIEIKDI) were synthesized by GenScript Corp. (NJ, USA) and NeoMPS SA (Strasbourg, France). Laminin was purified as previously described by (37). The group I metabotropic glutamate receptors agonist s(S)-3,5-Dihydroxyphenylglycine (DHPG), mGluR1 and 5 antagonists LY367385 and 2-methyl-6-(phenylethynyl)-pyridine (MPEP), respectively, were purchased from Tocris Biosciences (Ellisville, MO). Mitomycin C was purchased from Sigma. TO PRO3-iodide was purchased from Invitrogen. The antibodies used were mAb 3F4 (Dako), mouse polyclonal anti-PrP<sup>C</sup> antibody (33), mouse monoclonal anti-MAP2 (Sigma), rabbit anti-cleaved caspase 3 (Cell Signaling), goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 546 (Molecular Probes), anti-mouse IgG conjugated to R-phycoerythrin (Dako).

**Cell Culture**

CF10, a PrP<sup>C</sup>-null neural cell line derived from 129/Ola Prnp<sup>0/0</sup> mice (16) and immunoreactive for the neuroectodermal stem cell marker nestin (38; 39; 35; 40), was used for the reconstitution of PrP<sup>C</sup> expression. CF10 cells were cultured in DMEM (Invitrogen) containing glutamine (2 mM; Invitrogen), penicillin (100 IU), and streptomycin (100 µg/ml; Invitrogen) supplemented with 10% fetal bovine serum and cultured at 37°C and 5% CO<sub>2</sub>.

**Transfection, selection, and sorting**

We used a site-directed mutagenesis kit (Stratagene #200518) to make the following amino acid substitutions in the PrP<sup>C</sup> protein: P101L, P104L, A116V, D177N, V179I, and E199K using a pcDNA3 PrP3F4 wild-type vector (41). The sequences of the primers used for the mutagenesis are showed in Table 1. PCR was carried out according to the manufacturer’s recommendations: 1 µl (20U) of DpnI (New England Biolabs) was added to the plasmid DNA and incubated at 37°C for 1h to digest methylated DNA. All constructs were sequenced to confirm the presence of the desired mutations and the integrity of the rest of the sequence, and were used to transform competent E. coli JM109 cells. CF10 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions using a 1:3 ratio of DNA to Lipofectamine. Following transfection, cells were selected with G418 (2 mg/ml; Invitrogen) for 15 days, and resistant cells were subjected to magnetic cell sorting, using 3F4 antibody (Dako), magnetic micro beads, and magnetic separation columns (Miltenyi Biotec) Cells were maintained as a non-
clonal population. DNAs from all cell lines were also sequenced to confirm the presence of the desired mutations and the integrity of the rest of the PrP sequence.

**Flow cytometry**
To analyze PrP expression on the plasma membrane, transfected CF10 cells and non-transfected controls were incubated with blocking solution (0.5% BSA in PBS) plus 10µg/mL 3F4 antibody followed by anti-mouse IgG conjugated to R-phycoerythrin (1:200), both for 1h at 4°C. Analyses were performed using a FACS Calibur flow cytometer (BD Biosciences), and data acquired from 10,000 events were analyzed using CellQuest software (BD Biosciences).

**Immunofluorescence**
Cells were plated on glass coverslips, fixed with 4% paraformaldehyde in 0.12M sucrose in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. After rinsing with PBS, cells were blocked with PBS plus 5% BSA and incubated with 1µg/mL 3F4 antibody or mouse monoclonal anti-MAP2 (1:200) for 16h. Following washes in PBS, cells were incubated with the secondary antibodies (1:1000): anti-mouse Alexa Fluor 488 for MAP2 and 546 for PrP, also nuclear staining with 5µM TO PRO-3 (Invitrogen) for 1h. Coverslips were mounted on slides using Fluorsave Reagent (Calbiochem). Immunolabeled cells were imaged using confocal microscopy (Leica TCS SP5 II).

**Western blotting**
Cell extracts were prepared by homogenizing the cell pellet in lysis buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing a protease inhibitor mixture (complete protease inhibitor tablets; Roche Diagnostics). Proteins were resolved using SDS-PAGE, transferred to nitrocellulose membranes (Millipore), and incubated with 1µg/mL 3F4 antibody followed by HRP-anti-mouse IgG. Immune-reactivity was revealed by enhanced chemiluminescence (ECL Plus; GE Healthcare).

**Proteinase K resistance**
Transfected CF10 and control cells were washed twice with cold PBS, scraped from plate, pelleted by centrifugation, and lysed in cold buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS, 10 mM Tris-HCl, 10 mM NaCl and 10 mM EDTA). For proteolysis experiments, lysates were shared (25% for control and 75% for digestion) and incubated for 30 min at 4°C with proteinase K (PK) (Roche) at the concentrations indicated. Samples were precipitated with methanol. One volume of sample was mixed with 2.5 volumes of methanol. After 2 hr at -80°C, the mix was centrifuged at 1000 xg for 30 min at 4°C, the supernatant was discarded, and pellets were air dried. The dried pellets were dissolved in resuspension buffer (150 mM NaCl, 50 mM Tris-HCl, 50 mM EDTA). The presence of residual, proteinase K-resistant PrP was evaluated by western blotting using mouse anti-PrP antibody (1:1000; 33). To quantification, the values were relativized by the extract concentration.

**Calcium signaling and data analysis**
CF10 cells (transfected or non-transfected) were plated onto coverslips and serum-starved for 48h prior to the experiments. Cells were loaded with 10µM of the intracellular Ca^{2+} indicator Fluo3 AM (Invitrogen, Carlsbad-CA) for 30 min at 37°C in DMEM (Invitrogen) supplemented with 2mM CaCl_2. Cells were washed three times with PBS and resuspended in Krebs buffer (124mM NaCl, 4mM KCl, 25mM HEPES, 1.2mM MgSO_4, 10mM glucose) supplemented with 2mM CaCl_2. Ca^{2+}-free experiments were performed in Krebs buffer without CaCl_2 plus 1mM EGTA. Cells were pre-incubated in the presence or absence of mGluR1 and/or mGluR5 inhibitors LY367385 (50µM) or MPEP (5µM), respectively, for 30 min, followed by treatment with Ln-γ1 peptide (37µM). In some experiments, CF10 cells were treated with Ln-γ1 peptide (37µM) followed by the mGluR1/mGluR5 agonist DHPG (100µM). In control experiments, cells were also treated with the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (THG; 1µM). All experiments were done at 37°C. Image acquisition was
performed using confocal microscopy. The fluorescent signal was normalized as F1/F0 (F1, maximal fluorescence after drug addition and F0, basal fluorescence before drug addition). Software-based analysis (WCIF ImageJ, NIH) measured the change in the fluorescent signal in the selected cell as function of time. Experiments were carried out in at least three distinct cell cultures, and 40-50 cells were monitored in each experiment. Traces represent typical single cell responses.

Process outgrowth assay
CF10 cells (transfected and non-transfected) were pretreated with 10µg/mL Mitomycin C for 3h at 37ºC in a 5% CO₂ atmosphere. After washing, cells were detached with 0.02% EDTA in PBS and plated onto coverslips (12mm²) coated with 5µg/ml of poly-L-lysine plus 37µM BSA-Ln-γ1 peptide, 37µM BSA-SCR peptide or laminin and incubated by 48h at 37ºC and 5% CO₂ atmosphere. Cells were fixed with 4% paraformaldehyde in 0.12M sucrose in PBS for 20 minutes at room temperature, washed 3 times with PBS, and processed for immunocytochemistry. Morphometric analysis was performed using Image J software (National Institute of Health) and the Neuron J plug-in. The analyzed parameters consisted of the percentage of cells with cytoplasmic processes. A total of 200 cells were analyzed per sample.

Cell death assay
The effect of mitomycin C upon cell death was evaluated after 3h or 48 h of treatment using trypan blue exclusion or by activation of active caspase 3 respectively. For cell death protection assays, non-transfected CF10 cells, transiently transfected with cDNAs encoding a 3F4-tagged full-length murine PrP<sub>C</sub> (PrP<sub>3F4</sub>) or the PrP<sub>C</sub> mutants: 101L, 104L, 116V, 177N, 179I, and 199K which correspond to the disease-associated human mutations at amino acids 102, 105, 117, 178, 180, and 200, respectively. All the transfected PrP<sub>C</sub> proteins carried a methionine at amino acid 128. Transfected CF10 cells were selected for similar expression of either wild-type or mutated PrP<sub>C</sub> molecules, maintained as a non-clonal population, and verified by flow cytometry in non-permeabilized cells using 3F4 antibody (Fig. 1A), immunofluorescence of permeabilized cells (Fig. 1B), and western blotting (Fig. 1C). Wild-type and mutated PrP<sub>C</sub> expression was properly reconstituted in CF10 cells, and similar amounts of the proteins are present at the plasma membrane (Fig. 1A). However, as described previously (42; 43), the mutant, but not the wild-type, proteins displayed an intracellular accumulation (Fig. 1B). Western blot analysis also demonstrated the expression of di-, mono- and non-glycosylated forms of PrP<sub>C</sub>, with a molecular weight ranging from 24 to 38 kDa (Fig. 1C). As expected, no endogenous PrP<sub>C</sub> expression was present in non-transfected CF10 cells (Fig. 1A-C). The
localization of these proteins at the cell surface is of major importance to this study, as PrP<sup>C</sup> distribution at the membrane is crucial for laminin binding (24; 35).

**PrP<sup>C</sup> mutants show minor resistance to proteinase K digestion**

The TSEs are characterized by the conversion of the protease-sensitive prion protein into aggregates of protease-resistant isoform associated with the neuropathogenic process in vivo (44). Our results show that the proteinase K (PK) resistance of the 101L, 116V, 177N, and 199K mutants was essentially identical to that of wild-type PrP<sup>C</sup>. The 104L and 179I mutants have a slight increase in resistance to PK-mediated degradation compared to wild-type PrP<sup>C</sup> (Fig. 2). However, it is also possible that the apparent proteinase resistance of these mutants compared to wild-type proteins is caused by their relative insolubility in Triton-X100. These results are in agreement with similar findings for pathological PrP<sup>C</sup> mutants in different cell models (45; 46; 47; 48). However, it is important to note that the PK concentrations used in the current study are at least 5 times lower than those used to show resistance of the prion isoform associate with infection (49; 50; 51). Thus, suggesting that a putative function of these proteins may not be affected by aggregation.

**The ectopic expression of wild-type PrP<sup>C</sup> reconstitutes Ln γ1 peptide signaling in CF10 cells**

Our previous results demonstrated that the interaction of PrP<sup>C</sup> with the Ln γ1 peptide, in primary neuronal cultures, leads to the mobilization of Ca<sup>2+</sup> from intracellular stores, followed by Ca<sup>2+</sup> influx and the activation of PKC, and ERK1/2 (35). This PrP<sup>C</sup>-mediated transmembrane signaling is dependent on the activity of group I mGluR (mGluR1 and/or mGluR5) receptors, which were found to associate with PrP<sup>C</sup> (35).

A pharmacological approach was conducted in order to evaluate the activity of mGluR1 and/or mGluR5 in CF10 cells. The treatment with DHPG, a group I mGluR agonist, causes an increase in cytoplasmic calcium levels in non-transfected CF10 cells and those expressing wild-type PrP<sup>C</sup> (Figs. 3A, B respectively). This effect was partially impaired by LY367385 and MPEP, which are specific inhibitors of mGluR1 and mGluR5, respectively (Figs. 3F, H) and DHPG-mediated calcium increases were completely abolished upon treatment with both inhibitors (Fig. 3J). Thus, non-transfected and wild-type PrP<sup>C</sup>-transfected CF10 cells (PrP3F4) express functional mGluR1 and mGluR5.

When wild-type PrP<sup>C</sup>-transfected CF10 (PrP3F4) cells were treated with the Ln γ1 peptide, a significant increase in cytoplasmic calcium levels was observed (Fig. 3C). Conversely, no increases in intracellular Ca<sup>2+</sup> were induced by Ln γ1 application in non-transfected PrP-null CF10 cells. These cells have normal levels of intracellular store calcium, as evidenced by their response to thapsigargin (THG; Fig. 3D). Treatment with LY367385 (Fig. 3E) or MPEP (Figs 3G) partially inhibited intracellular Ca<sup>2+</sup> mobilization by Ln γ1 peptide in wild-type PrP<sup>C</sup>-transfected cells, while a combination of the two inhibitors completely abolished the Ln γ1 peptide induced increases in the calcium signal (Fig. 3I). Quantitative analyses of these data are presented in Figure 3K. These results are in accordance with our previous findings in primary hippocampal neurons (35) and indicate that the expression of wild-type PrP<sup>C</sup> in PrP<sup>C</sup>-null neuronal CF10 cells is able to reconstitute the cellular signaling modulated by the PrP<sup>C</sup>- Ln γ1-mGluR1/5 pathway.

**CF10 cells expressing mutant prion proteins present impaired Ln γ1-induced calcium signaling**

Since calcium is one of the major intracellular signals triggered by PrP<sup>C</sup>-Ln γ1 peptide-mGluR1/5, we then examined whether disease-related mutations in the PrP<sup>C</sup> protein cause disturbances in Ln γ1 peptide-induced intracellular calcium signaling. As previously demonstrated (Fig. 3C), the expression of wild-type PrP<sup>C</sup>, PrP3F4, rescued Ca<sup>2+</sup> signaling triggered by Ln γ1 peptide (Fig. 4A) in CF10 cells. Interestingly, the expression of PrP<sup>C</sup> proteins carrying TSE-related mutations only partially rescued Ln γ1 peptide...
induced Ca\textsuperscript{2+} signaling in CF10 cells when compared to cells expressing the wild-type protein (Fig. 4C-H, I). In addition, when treated with DPH all cells bearing mutant proteins show an increment in calcium signaling similar to those expressing wild-type protein (Fig. 4J), indicating that they have similar levels and activity of mGluR1/5. These data indicate that TSE-associated PrP\textsuperscript{C} mutations result in deficiencies in intracellular calcium mobilization triggered by Ln γ1 peptide.

**PrP\textsuperscript{C} mutations impaired process outgrowth triggered by Ln γ1 peptide**

As previously demonstrated (35), the association of PrP\textsuperscript{C} and the Ln γ1 peptide co-opts mGluR1/5 to promote neuronal differentiation and neuritogenesis in primary neuronal cultures through the mobilization of intracellular Ca\textsuperscript{2+}. CF10 cells are strongly positive for nestin (36) and neuron-specific enolase, but are negative for βIII tubulin and NeuN (data not shown), thus, it is likely that they are neural progenitor cells. In addition, CF10 cells were immobilized via expression of the SV40 large T antigen (Ln), a powerful molecule associated with neurite extension (52; 53). Even treatment with the full-length laminin (Ln), a powerful molecule associated with neurite extension (54), was unable to promote process outgrowth in CF10 cells expressing (PrP3F4) or without PrP\textsuperscript{C} expression (Fig. 5A). To promote cell cycle arrest, CF10 cells were treated with Mitomycin C, a well-known inhibitor of cell proliferation in vitro (55; 56; 57). We found that, at the concentration used, Mitomycin C impaired cell growth (Fig. 5B) without affecting cell viability (Fig. 5C). Morphometric analyses showed that Mitomycin C-treated CF10 cells, either non-transfected or transfected with wild-type PrP\textsuperscript{C} (PrP3F4) extended robust cytoplasmic processes when plated onto full-length laminin (Fig. 5D). Nonetheless, these cells do not express βIII tubulin and NeuN (data not shown) after this treatment. Yet, under specific experimental conditions, these cells are able to growth neurite-like processes; in addition, when full-length Ln is present other receptors besides PrP\textsuperscript{C}, probably integrins, may contribute to the phenotype. In contrast, process outgrowth was observed when wild-type PrP\textsuperscript{C}-expressing cells (PrP3F4), but not the non-transfected CF10 one, was plated onto Ln γ1 peptide (Fig. 5D). These results confirm that the outgrowth of neurite-like processes, mediated by the PrP\textsuperscript{C}-Ln γ1 interaction, can be rescued in CF10 cells expressing the wild-type PrP\textsuperscript{C}.

In CF10 cells that express mutated PrP\textsuperscript{C}, Mitomycin C also inhibited cell growth (Fig. 6A) without affecting viability measured by trypan blue exclusion (Fig. 6B). Cell death induced by Mitomycin C 30 h after treatment was also evaluated by activation of caspase 3. Contrary to the positive control, staurosporin, Mitomycin C did not induce cell death (Fig. 6C). These cells, together with non-transfected and wild-type PrP\textsuperscript{C}-expressing CF10 cells (PrP3F4), were also evaluated for process outgrowth mediated by the Ln γ1 peptide. **Figure 6E** shows representative images of the morphology of these cells following Ln γ1 peptide treatment and immunolabeling with microtubule-associated protein 2 (MAP2). CF10 cells transfected with wild-type PrP\textsuperscript{C} (PrP3F4) presented long cytoplasmic processes (arrows in first panel; upper left). Remarkably, the addition of the Ln γ1 peptide was unable to promote process outgrowth in CF10 cells expressing mutated PrP\textsuperscript{C} molecules (quantification in Fig. 6D). These results indicate that CF10 cells lacking PrP\textsuperscript{C}, or expressing mutated PrP\textsuperscript{C}, are unable to extend cytoplasmic process upon Ln γ1 peptide treatment.

**PrP\textsuperscript{C} mutations impaired protection against cell death triggered by Ln γ1 peptide**

PrP\textsuperscript{C} has been involved in neuronal protection against cell death and consensus results from different groups showed that neurons from PrP\textsuperscript{C}-null mice presented a higher sensitivity to agents that induce cell death, including staurosporin, than neurons from wild-type animals (reviewed by 13). Remarkable, our results (Fig. 6C) demonstrated that CF10 cells or cells expressing mutated PrP\textsuperscript{C} also presented a
higher sensitivity to staurosporine than cells that were reconstituted with wild-type PrP<sup>c</sup>. We then tested whether Ln γ1 chain peptide induce protection against staurosporin-induced cell death. Ln γ1 peptide, but not the scrambled peptide (SCR), was able to protect PrP<sub>3F4</sub> cells against staurosporin-induced cell death. However, any protective effect was observed when CF10 or CF10 cells expressing PrP<sup>c</sup> mutants were cultured on Ln γ1 peptide (Fig. 7A).

Finally, some experiments were conducted to evaluate if mutant PrP<sup>c</sup> molecules present dominant negative or prion effects upon wild-type PrP<sup>c</sup>. CF10, PrP<sub>3F4</sub> and CF10 cells expressing the mutants 101L and 179I were transiently transfected with PrP<sub>3F4</sub> (Fig. 7B) and tested for Ln γ1 protection against cell death induced by staurosporin. The expression of PrP<sub>3F4</sub> was able to rescue the neuroprotective effects of Ln γ1 even when in the presence of mutated PrP<sup>c</sup> molecules (Fig. 7C). Thus, indicating that these mutants do not have a dominant negative or prion effect upon wild-type PrP<sup>c</sup> and may have lose their function triggered by engagement with Ln γ1 peptide.

**DISCUSSION**

The goal of the current study was to investigate the consequences of PrP<sup>c</sup> loss-of-function that result from disease-associated mutations in the PrP<sup>c</sup> protein. To model the function of PrP<sup>c</sup> we expressed wild-type and mutant PrP<sup>c</sup> proteins on a PrP<sup>c</sup>-null background neural cell line (CF10). Our results show that substitutions within the PrP<sup>c</sup> protein decrease calcium signaling and impair process outgrowth and protective response that are mediated by the interaction between PrP<sup>c</sup> and Ln γ1 chain peptide.

The effects of PrP<sup>c</sup> and PrP<sup>c</sup> mutants expression have been previously investigated at the cellular level using neuroblastoma, breast cancer, fibroblasts and Fischer rat thyroid cell lines (43; 58; 59; 60; 61). However, data regarding the cellular and biochemical properties of prion proteins are controversial; disparities between groups may reflect differences in the cell types in which these proteins have been expressed (21; 27; 62; 63; 64). In the current application, we have addressed this question in non-tumor-derived neural cells, a model may more faithfully replicate the effects of prion proteins in neurons. Remarkable, the reconstitution of wild-type PrP<sup>c</sup> in PrP<sup>c</sup>-null neural CF10 cells was able to reproduce all the effects that have been observed in primary neuronal cultures following the interaction of PrP<sup>c</sup> with stress inducible protein 1 (STI1) (39) and with Ln γ1 chain peptide (33).

The current study also demonstrates the utility of the CF10 cell lines as a model for the study of exogenously-expressed prion mutated proteins. We found that these cells expressed PrP<sup>c</sup> at the cell surface, where it should be to interact with ligands at the membrane or at the extracellular matrix (including Ln) and whose interaction modulates cellular functions (13). In addition, transfected CF10 cells express similar levels of the wild-type or mutated PrP<sup>c</sup> at the cell membrane, although some intracellular accumulation of the mutant PrP<sup>c</sup> proteins was detected. Moreover, only a minor PK-resistance was observed in 2 of 6 PrP<sup>c</sup> mutants suggesting that aggregation may not interfere with the function of these proteins.

PrP<sup>c</sup> binds to the carboxy-terminal domain of the Ln γ1 chain, a region where no others laminin receptors have been mapped (24). Thus, the use of the Ln γ1 peptide allows the researcher to specifically examine phenotypes associated with the PrP<sup>c</sup>-Ln interaction (24). We recently demonstrated that the Ln γ1 peptide induces PrP<sup>c</sup>-dependent neuritogenesis via increases in intracellular Ca<sup>2+</sup>, and the activation of PKC and ERK1/2 in a manner dependent upon the participation of mGluR1/5, which also interact with PrP<sup>c</sup> (35).

Our data show that calcium signaling mediated by the Ln γ1 peptide and mGLUR1/5 is partially impaired in cells expressing the 101L, 104L, 116V, 177N, 179I, and 199K PrP<sup>c</sup> mutants, compared to wild-type PrP<sup>c</sup>. Remarkably, in the course of TSE disease, it has been demonstrated that prion infection modifies Ca<sup>2+</sup> responses (65) and impairs mGluR1/PLC/PKC pathway signaling in...
neurons derived from a murine model of BSE, as well as humans with sporadic CJD (66; 67). Together, these results suggest that modifications in the PrP^C protein impact type 1 mGluR-mediated cell signaling pathways, and that these alterations play a role in both sporadic and genetic prion diseases.

Although only a partial decrease on the Ln γ1-mediated Ca^{2+} response was observed in cells expressing mutant PrP^C proteins, we found that Ln γ1-induced process outgrowth was completely abolished in the presence of mutant PrP^C molecules. One simple explanation is that the increment in Ca^{2+} levels did not achieve the threshold necessary to promote this phenotype in these cells. It is important to note that the 177N and 179I mutants are in the vicinity of the Ln γ1 binding site on the PrP^C molecule, amino acids 173-182 (22). Thus, these alterations may abrogate the interaction between PrP^C and laminin. On the other hand, we show similar results with PrP^C molecules carrying disease-association mutations in the amino-terminal domain (101L, 104L, and 116V) or in the vicinity of the third α-helix region (199K). It is possible that these mutations instead affect the interaction between PrP^C and mGluR1/5; however, the binding site between these molecules has not yet been identified.

PrP^C was also pointed to protect neurons against injury both in vitro and in vivo (13; 19; 25). PrP^C protein is itself a receptor for the secreted form of STI1, an interaction which has been shown to promote neuroprotection and neuronal differentiation (25, 68, 69). Our present data indicate that Ln γ1 peptide impaired staurosporine-induced cell death in PrP3F4 cells while Ln γ1 peptide fails to rescue CF10 and CF10 cells expressing PrP^C mutants from death. Indeed, the results are consistent with the participation of the Ln γ1 peptide-PrP^C complex in neuroprotection and that mutations within PrP^C abolish its neuroprotective function. Furthermore, when the wild-type PrP^C is co-expressed with mutant PrP^C molecules, Ln γ1 peptide rescues staurosporine-induced cell death. This seems to show the absence of a dominant-negative effect, which is consistent with previous data showing that neurodegeneration mediated by expression of PrP^C deletion mutants (some of the deleted sites include amino acids mutated here) could be reversed by expression of the wild-type protein (70, 71, 72).

The results of the current study may provide support to the idea that PrP^C plays an important role as a scaffolding protein that has allosteric functions and dysfunctions (36). The interaction between PrP^C and STI1, or PrP^C and the STI1 peptide 230-245, which mimics the PrP^C binding site, has been shown to alter the structure of both components, potentially impacting the ability of either protein to interact with other ligands (73) and to organize a functional signaling platform (13). The conformational alterations imposed by the PrP^C Ln γ1 interaction remain unknown. However, some PrP^C mutants have been shown to destabilize the native PrP^C structure, possible by increasing the stability of partially folded intermediate species (74; 75; 76; 77). The conformational alterations imposed by these mutations may alter the ability of PrP^C to interact with ligands such as laminin or mGluR1/5, and may even impair the interactions of these ligands with other proteins. Depending on the multi-complex formed, a specific cellular event could be partially impaired, as is observed for Ca^{2+} signaling, or completely blocked, as is the case for process outgrowth, by mutations in PrP^C.

PrP^C mutated molecules may lose the ability to modulate Ln γ1-mediated neuronal plasticity and survival, thus representing the loss of a physiological function associated with the PrP^C. The current study points towards the necessity of further evaluation of the role of native and mutated PrP^C proteins in neuronal plasticity in animal models of genetic prion diseases, even prior to the onset of clinical symptoms.
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FOOTNOTES

Competing Interests
The authors declare no actual or potential conflicts of interest with other people or organizations in the present work.

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FIGURE LEGENDS

**TABLE 1. List of primer sequences.** Primers used to perform the site-directed substitutions of amino acids P101L, P104L, A116V, D177N, V179I, and E199K on pcDNA3 PrP<sub>C</sub> wild-type PrP<sup>C</sup> (I28M). Letters in italics denote the substituted nucleotides.

**FIGURE 1. Expression of wild-type and mutant forms of PrP<sup>C</sup> in PrP<sup>C</sup>-null neural cells.** The PrP<sup>C</sup>-null neuronal cell line CF10 was transfected with an expression vector encoding wild-type PrP<sup>C</sup> (PrP3F4) or PrP<sup>C</sup> mutants 101L, 104L, 116V, 177N, 179I, and E199K and maintained as a non-clonal population. PrP<sup>C</sup> expression was detected using the anti-3F4 and R-phycoerythrin conjugated secondary antibody. **A.** Flow cytometry of non-permeabilized cells expressing wild-type or PrP<sup>C</sup> mutants. **B.** Immunofluorescence for PrP<sup>C</sup> in permeabilized cells using anti-3F4 antibody (red) and DAPI (blue). **C.** Western blotting of cell extracts using the anti-3F4 antibody.
**FIGURE 2.** PrP<sup>C</sup> mutants show minor resistance to proteinase K digestion. Extracts from CF10 cells expressing wild-type (PrP<sub>3F4</sub>) or PrP<sup>C</sup> mutants were treated or not with increasing concentrations of proteinase K (PK; 0, 0.5, 1 and 4 µg/mL) for 30 min at 4°C. The condition where cells were treated with PK had 3 times more total protein than the undigested condition. The remaining undigested PrP<sup>C</sup> was detected by western blotting (anti-PrP<sup>C</sup> antibody). A. The relative levels of PrP<sup>C</sup> represent the ratio between the levels of PrP<sup>C</sup> after PK treatment and the total load of PrP<sup>C</sup> (control). Relative values are represented as mean ± SE (n=3). B. Representative western blots contain 3 times more total protein in the PK digestion conditions than in non-digested ones. The asterisk on the left side of the panel is to show the migration of the molecular marker of 30 kDa.

**FIGURE 3.** mGluR1 and mGluR5 mediate intracellular calcium increase upon PrP<sub>C</sub>-Ln γ1 peptide binding. A. CF10 cells expressing PrP<sup>E</sup> wild-type (PrP<sub>3F4</sub>) and their PrP<sup>E</sup>-null counterpart (CF10), were loaded with 10µM Fluo3 AM and treated with the mGluR agonist DHPG (100µM). C, D. Ln γ1 peptide (37µM) or thapsigargin (THG) (1µM) in a medium supplemented with 2 mM CaCl<sub>2</sub>. E, F. Cells were also treated with Ln γ1 peptide or DHPG in the presence of mGluR1 antagonist (LY367385, 50µM). G, H. mGluR5 antagonist (MPEP, 5µM) or I, J. in the presence of both 50 µM LY367385 and 5 µM MPEP. K. Relative intracellular calcium levels are expressed as mean ± SE (n=3), (*p < 0.05 vs. control; **p < 0.001 vs. control; #p < 0.001 vs. PrP3F4 treated with Ln γ1 peptide; § p < 0.001 vs. CF10 treated with DHPG).

**FIGURE 4.** Partial impairment in calcium signaling mediated by Ln γ1 peptide in CF10 expressing mutant PrP<sub>C</sub>. CF10 cells transfected with A. wild-type PrP<sup>E</sup> (PrP<sub>3F4</sub>), B. non-transfected (CF10), or transfected with the PrP<sup>C</sup> mutants C. 101L, D 104L, E. 116V, F. 177N, G. 179I, and H. 199K were loaded with 10 µM Fluo-3 AM and treated with Ln γ1 peptide (37µM) and THG (1µM) in medium supplemented with 2mM CaCl<sub>2</sub>. I. Relative intracellular calcium levels are expressed as mean ± SE (n=7), (*p < 0.05; **p < 0.01). J. Intracellular calcium concentration in cells treated or not with DHPG. Calcium levels at untreated condition (white bars) were considered =1 and the values after DHPG treatment (gray bars) were relative to it. Values represent mean ± SE (n=3).

**FIGURE 5.** Process outgrowth mediated by the Ln γ1-peptide can be rescued following the reconstitution of PrP<sub>C</sub> expression in CF10 cells. A. CF10 cell lines with and without transfections of wild-type PrP<sup>E</sup> (PrP<sub>3F4</sub>) were cultured 48h on coverslips pretreated with 10µg/mL of full-length laminin or 5 µg/mL of poly-L-lysine. Cells were fixed, labeled, and process outgrowth was quantified. B, C. PrP3F4- and non-transfected CF10 cells were treated with 10µg/mL Mitomycin C or vehicle for 3 hours, washed and plated on poly-L-lysine for 48 h to assess: B. total cell number (proliferation) after 48 h or C. cell death (using trypan blue) after 3 h of Mitomycin treatment. Values represent mean ± SE (n=3), (*p<0.05 vs. control). D. PrP3F4- and non-transfected CF10 cells pretreated with 10 µg/mL Mitomycin C for 3 h were cultured on 5 µg/mL poly-L-lysine, poly-L-lysine plus laminin (2.5µg/mL or 5µg/mL), or poly-L-lysine plus Ln γ1 peptide (16.8µM or 37µM) for 48 h. Values represent mean ± SE (n=3), (*p<0.05 compared with the control PrP3F4 or CF10 in poly-L-lysine; **p<0.05 compared with the control PrP3F4 in poly-L-lysine).

**FIGURE 6.** Process outgrowth induced by Ln γ1 is impaired in cells expressing PrP<sub>C</sub> mutants. PrP3F4- and non-transfected CF10 cells, as well as CF10 cells expressing the PrP<sub>C</sub> mutants 101L, 104L, 116V, 177N, 179I, and 199K were treated with 10 µg/mL Mitomycin or vehicle for 3 hours washed and plated on poly-L-lysine for 48 h. A. total cell number (proliferation) (*p<0.05 vs. control). B. Percentage of cell death (using trypan blue) measured after 3h of Mitomycin C treatment (*p<0.05 vs control). C. Percentage of cell death (capase-3 activation) using staurosporine as positive control after 48h of Mitomycin C treatment (*p < 0.05 vs. control; *p < 0.05 PrP3F4 staurosporine treatment vs. all others staurosporine treatment).
D. Cells were treated with 10 µg/mL Mitomycin C for 3 hours, washed, plated on poly-L-lysine and treated with 37µM Ln γ1 scrambled peptide (SCR) or 37µM Ln γ1 peptide (γ1) for 48 h. Percentage of cells with process outgrowth was evaluated (*p < 0.05 vs al others).

E. Representative images of CF10 cells expressing wild-type (PrP3F4) and PrP<sup>C</sup> mutants treated with Mitomycin C followed by treatment with 37µM Ln γ1 peptide. The arrows show the structures considered as typical process outgrowth. For all experiments values represent mean ± SE of at least three independent experiments.

**FIGURE 7.** Cell survival promoted by Ln γ1 is impaired in cells expressing PrP<sup>C</sup> mutants. PrP3F4- and non-transfected CF10 cells, as well as CF10 cells expressing PrP<sup>C</sup> mutants 101L, 104L, 116V, 177N, 179I, and 199K were plated on 5 µg/mL poly-L-lysine plus 37µM Ln γ1 peptide (γ1) or 37µM Ln γ1 scrambled peptide (SCR) and treated or not with 100nM staurosporine. A. Quantification of active caspase 3 positive cells (* p<0.01 Ln γ1 peptide plus staurosporine treated CF10 and PrP<sup>C</sup> mutants vs. Ln γ1 peptide plus staurosporine treated PrP3F4). B. PrP3F4, CF10 cells, and CF10 cells expressing the PrP<sup>C</sup> mutants 101L and 179I were co-transfected with wild-type PrP<sup>C</sup> (PrP3F4) and immunobloted for PrP<sup>C</sup>. C. Cells were plated on 5 µg/mL poly-L-lysine plus 37µM Ln γ1 peptide or 37µM Ln γ1 scrambled peptide (SCR) and treated or not with 100nM staurosporine. Active caspase 3 positive cells were quantified (* p<0.01).

**Table 1**

| Primers | Sequences |
|---------|-----------|
| **Forward** P101L | 5’ CAG TGG AAC AAG **CTC** AGC AAA CCA AAA 3’ |
| **Reverse** P101L | 5’ TTT TGG TTT GCT **GAG** CTT GTC CCA CTG 3’ |
| **Forward** P104L | 5’ AAG CCC AGC AAA **CTA** AAA ACC AAC CTC 3’ |
| **Reverse** P104L | 5’ GAG GTT GGT TTT **TAG** TTT GCT GGG CTG 3’ |
| **Forward** A116V | 5’ GCA GGG GCT GCG **GTA** GCT GGG GCA GTA 3’ |
| **Reverse** A116V | 5’ TAC TGC CCC AGC **TAC** CGC AGC CCC TGC 3’ |
| **Forward** D177N | 5’ AAC TTC GTG CAC **AAC** TGC GTC AAT ATC 3’ |
| **Reverse** D177N | 5’ GAT ATT GAC GCA **GTT** GTG GAC GAA GTT 3’ |
| **Forward** V179I | 5’ GTG CAC GAC TGC **ATC** AAT ATC ACC ATC 3’ |
| **Reverse** V179I | 5’ GAT GGT GAT ATT **GAT** GCA GTC GTG CAC 3’ |
| **Forward** E199K | 5’ GAG AAC TTC ACC **AAG** ACC GAT GTG AAG 3’ |
| **Reverse** E199K | 5’ CTT CAC ATC GGT **CTT** GGT GAA GTT CTC 3’ |
Figure 1
Figure 3

A) PrP3F4
B) CF10
C) PrP3F4
D) CF10
E) PrP3F4 + LY
F) CF10 + LY
G) PrP3F4 + MPEP
H) CF10 + MPEP
I) PrP3F4 + LY + MPEP
J) CF10 + LY + MPEP

K)

PrP3F4
CF10
DHPG
γ1
LY
MPEP

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Figure 5
Disease-associated mutations in the prion protein impair laminin-induced process outgrowth and survival
Cleiton F. Machado, Flavio H. Beraldo, Tiago G. Santos, Dominique Bourgeon, Michele C. Landemberger, Martin Roffe and Vilma R. Martins

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