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Regulation of Amyloid β Oligomer Binding to Neurons and Neurotoxicity by the Prion Protein-mGluR5 Complex*[S]

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The prion protein (PrP^C) has been suggested to operate as a scaffold/receptor protein in neurons, participating in both physiological and pathological associated events. PrP^C, laminin, and metabotropic glutamate receptor 5 (mGluR5) form a protein complex on the plasma membrane that can trigger signaling pathways involved in neuronal differentiation. PrP^C and mGluR5 are co-receptors also for β-amyloid oligomers (AβOs) and have been shown to modulate toxicity and neuronal death in Alzheimer's disease. In the present work, we addressed the potential crosstalk between these two signaling pathways, laminin-PrP^C-mGluR5 or AβO-PrP^C-mGluR5, as well as their interplay. Herein, we demonstrated that an existing complex containing PrP^C-mGluR5 has an important role in AβO binding and activity in neurons. A peptide mimicking the binding site of laminin onto PrP^C (Ln-γ1) binds to PrP^C and induces intracellular Ca^{2+} increase in neurons via the complex PrP^C-mGluR5. Ln-γ1 promotes internalization of PrP^C and mGluR5 and transiently decreases AβO biding to neurons; however, the peptide does not impact AβO toxicity. Given that mGluR5 is critical for toxic signaling by AβOs and in prion diseases, we tested whether mGlur5 knock-out mice would be susceptible to prion infection. Our results show mild, but significant, effects on disease progression, without affecting survival of mice after infection. These results suggest that PrP^C-mGluR5 form a functional response unit by which multiple ligands can trigger signaling. We propose that trafficking of PrP^C-mGluR5 may modulate signaling intensity by different PrP^C ligands.

The prion protein (PrP^C)[3] was originally discovered as a substrate for prion disease propagation in mammals (1). Several studies on PrP^C physiological function revealed dozens of PrP^C partners (2–4). In neurons, PrP^C interactions with some of these ligands trigger multiple effects, including regulation of protein synthesis, differentiation, neuroprotection, and neuritogenesis (4–17). Given that PrP^C is a glycosylphosphatidylinositol (GPI)-anchored protein, signal transduction requires the formation of complexes between PrP^C and transmembrane receptors. PrP^C interactions have been demonstrated for α7 nicotinic acetylcholine receptor (5), group I metabotropic glutamate receptors (10, 18, 19), ionotropic glutamate receptors (20, 21) and purinergic receptors (22). These results suggest that PrP^C functions as an extracellular scaffolding protein, able to organize multiprotein complexes at the cell surface (3, 4). Increasing evidence indicates that such scaffolding can be neurotoxic. For instance, when PrP^C binds oligomeric forms of β-amyloid peptides (AβO) (23), mal-adaptive signaling via metabotropic glutamate receptor 5 (mGluR5) is elicited (18), which initiates multiple changes in synaptic homeostasis, leading to excitotoxicity, endoplasmic reticulum stress, and eventually to synaptic degradation and neuronal cell death (18, 23–30). Thus, efforts have been made to disrupt this complex and downstream pathways to prevent neurotoxic consequences. Antibodies specific to certain PrP^C regions were efficient in preventing AβO binding to the neuronal surface (31–33), but such antibodies can also trigger toxic neuronal signaling by themselves (34), mediated by PrP^C N terminus domain (35). The PrP^C N-terminal domain, known as N1 peptide, which is usually secreted into extracellular space by α-secretase action on PrP^C, also exhibited a neuroprotective

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[S] This article contains supplemental Figs. S1–S3 and supplemental Experimental Procedures.

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[3] The abbreviations used are: PrP^C, cellular form of PrP; PrP, prion protein; PrP^{Sc}, misfolded form of PrP; GPI, glycosylphosphatidylinositol; Aβ, β-amyloid; AβO, β-amyloid oligomer; mGluR, metabotropic glutamate receptor; Ln, laminin; SCR, scrambled; STI1, Stress-inducible phosphoprotein 1; KRH, Krebs-Ringer buffer.

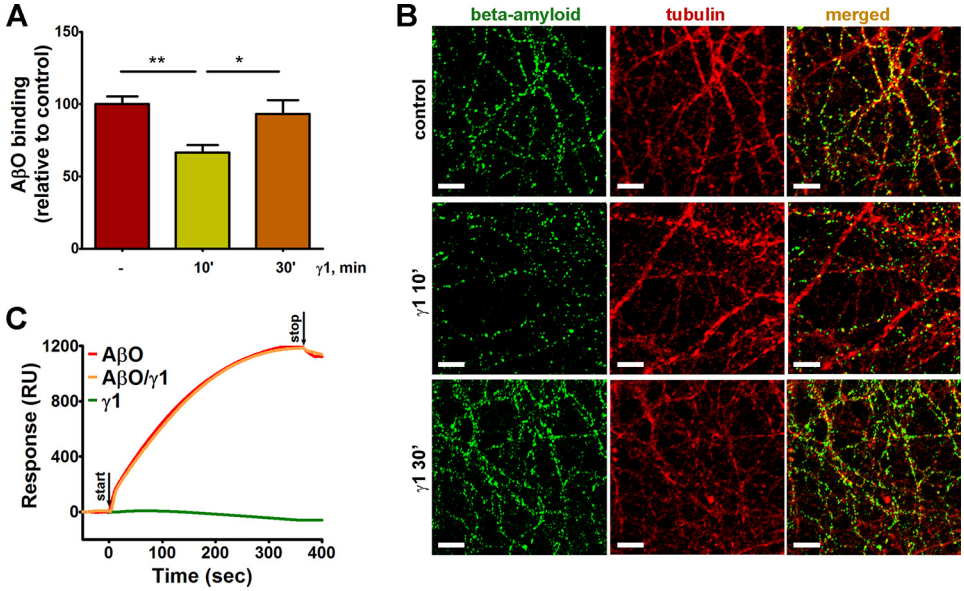

FIGURE 1. **Ln-γ1 effect on AβO binding to neurites and on-chip PrP.** *A*, levels of the neurite-bound AβOs decrease in cultures treated with Ln-γ1 peptide (RNIAEIIKDI) for 10, but not for 30 min. *B*, representative images of hippocampal neuronal cultures, immunostained for β-amyloid (*green*) and tubulin (*red*), as described under "Experimental Procedures." *Scale bar* = 10 μm. *C*, SPR kinetics of ligands binding to PrP on a Ni-nitrilotriacetic acid chip. Ln-γ1 (*green*) or AβOs (*red*) were injected separately or simultaneously (*orange*). *RU*, response units.

effect, presumably due to the sequestration of AβOs from the extracellular space (36, 37).

Stress-inducible phosphoprotein 1 (STI1), a secreted co-chaperone that can bind and activate PrP^C-dependent signaling, has been shown to prevent the toxicity of AβOs in cultured neurons and brain slices (38). mGluR5 ligands (antagonists and modulators) have also been shown to impact on AβO toxicity and cognitive deficits (18, 25, 29, 39, 40). The interaction between laminin γ1 chain and PrP^C is of particular interest, given that the PrP^C-mediated signaling triggered by laminin γ1 chain-mimicking peptide (1575–1584, Ln-γ1) depends on mGluR5 (10). Of importance, Ln-γ1 signaling via the complex PrP^C-mGluR5 leads to neuritogenesis (10), a process known to be disrupted by AβO-induced toxicity (41, 42).

Here we investigated the relationship between Ln-γ1, PrP^C, and mGluR5 in AβO toxicity and the role of mGluR5 in prion infection. Our data suggest that a preformed complex containing mGluR5 and PrP^C cooperates for Aβ oligomer binding to neurons, and that Ln-γ1 is ineffective to prevent AβO-induced toxicity. Moreover, mGluR5 may also regulate some of the toxicity related to prion infection.

Results

Ln-γ1 Modulates AβO Binding to Neurites—There is accumulating evidence that PrP^C binds AβOs on the neuronal surface, initiating toxic signaling pathways (18, 23, 27, 33, 38). Previously, we showed by multiple assays that a PrP^C ligand, STI1, decreases AβO binding to the prion protein and prevents neuronal death induced by AβOs (38). Given that similarly to AβOs, Ln-γ1 also engages PrP^C and mGluR5, we tested whether the Ln-γ1-dependent activation of PrP^C could affect its binding to AβO and toxicity. We observed a 30% decrease in neurite-bound AβOs when neuronal cultures were pretreated with Ln-γ1 laminin peptide for 10 min (Fig. 1, *A* and *B*). Surprisingly, there were no changes in AβO binding when neuronal cultures were pretreated with Ln-γ1 peptide for 30 min (Fig. 1, *A* and *B*). To further understand the potential mechanisms by which Ln-γ1 interferes with Aβ biding to PrP^C, we performed an SPR study (Fig. 1*C*). Likely due to its small size, the Ln-γ1 peptide did not produce any signals on SPR (Fig. 1*C*), albeit each preparation was able to cause PrP^C-dependent calcium signal in neurons, as described previously (10). Importantly, Ln-γ1 peptide was unable to interfere with AβO binding (Fig. 1*C*). These results suggest that treatment with Ln-γ1 peptide decreases AβO binding in live cells by a mechanism distinct from competition with AβO for PrP^C binding sites.

Ln-γ1 Induces Decrease in Cell Surface PrP^C—To investigate the potential mechanisms by which Ln-γ1 peptide could modulate PrP^C, we incubated neuronal CF10 cells (a PrP^C-null cell line) stably transfected with PrP^C with Ln-γ1 peptide and measured cell surface PrP^C by biotinylation. Treatment of cells with Ln-γ1 for 10 min significantly decreased cell surface PrP^C by 30–40%, which then returned to normal levels after 30 min of Ln-γ1 treatment (Fig. 2*A* and *B*). This result was confirmed by confocal and total internal reflection fluorescence imaging of HEK293T cells transfected with PrP^C fused to GFP, suggesting that Ln-γ1 peptide treatment causes internalization of PrP^C (supplemental Experimental Procedures and Fig. S1). Ln-γ1 peptide triggers calcium signaling via PrP^C-mGluR5 complex; hence it is possible that the changes in cell surface PrP^C levels may reflect a desensitization mechanism. Treatment of PrP^C-CF10 cells with Ln-γ1 peptide led to a significant increase in intracellular Ca^{2+}, but a second application of the peptide 10 min after the first treatment failed to elicit a second bout of Ca^{2+} signaling (Fig. 2, *C* and *D*). The same pattern of intracellular Ca^{2+} release was observed in neurons treated with Ln-γ1 peptide (Fig. 2, *E* and *G*). In contrast, when Ln-γ1 peptide was reapplied 30 min after the initial treatment, a robust increase in intracellular Ca^{2+} was observed (Fig. 2, *F* and *G*). Thapsigargin

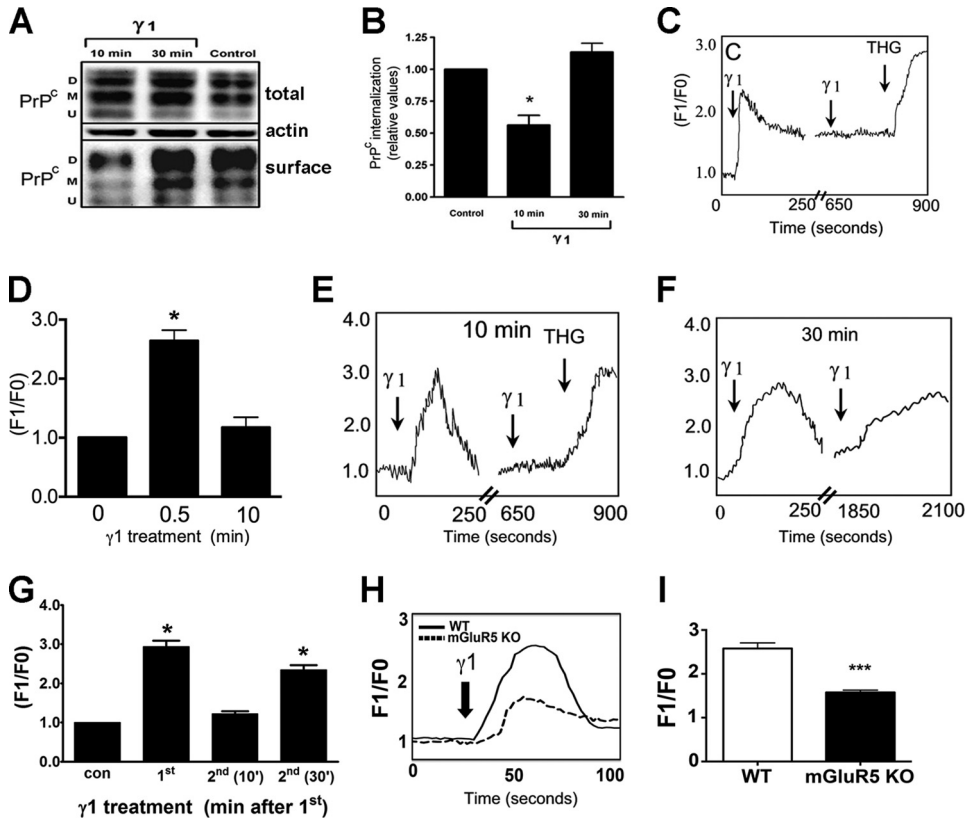

FIGURE 2. **Effect of Ln-γ1 treatment on PrP^C localization and intracellular calcium in cells.** *A* and *B*, Ln-γ1 induces transient PrP^C internalization in CF10 cells. *A*, representative Western blotting image of diglycosylated (*D*), monoglycosylated (*M*) and unglycosylated (*U*) forms of PrP^C. *B*, quantification of surface/total protein levels from at least 3 independent experiments. *C* and *D*, Ln-γ1 induces an increase in intracellular Ca^{2+} levels in CF10 cells, and repeated administration of Ln-γ1 over 10 min has no effect. *C*, calcium response (relative Fluo-4 fluorescence kinetics) induced by Ln-γ1. Thapsigargin (1 μM) (*THG*) was used as a positive control for intracellular calcium mobilization. *D*, quantification of Ca^{2+} levels, averaged from at least 60 cells measured in at least 3 independent experiments. *E–G*, Ln-γ1 induces increase in intracellular Ca^{2+} levels in primary hippocampal neurons. *E*, relative Fluo-4 fluorescence kinetics of the initial Ln-γ1 treatment and the repeated one in 10 min. *F*, the same for the repeated γ1 treatment in 30 min after the initial one. *G*, quantification of Ca^{2+} levels, averaged from at least 60 cells measured in at least 3 independent experiments. *H* and *I*, Ln-γ1-induced increase in intracellular Ca^{2+} in wild-type and mGluR5$^{-/-}$ neurons. *H*, relative Fluo-4 fluorescence kinetics. *I*, quantification of Ca^{2+} signal amplitude, averaged from at least 60 cells measured in at least 3 independent experiments. *Error bars* indicate mean ± S.E. *, $p < 0.05$; ***, $p < 0.001$.

(1 μM), a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which stimulates intracellular calcium mobilization, was used as a positive control (43). These results suggest that about 30–40% of cell surface PrP^C is available for signaling by Ln-γ1 peptide, and when this pool of PrP^C is internalized, the remaining cell surface protein cannot trigger calcium signaling.

We have shown previously that mGluR5 antagonists blocked the intracellular calcium signal induced by Ln-γ1/PrP^C interaction (10). To further test the role of these two proteins, we used hippocampal cell cultures from mouse embryos lacking either mGlur5 or PrP^C or both. Ln-γ1 peptide-induced calcium release is significantly decreased in mGluR5$^{-/-}$ neurons (Fig. 2*H*). The residual signal probably depends on mGluR1, which also was shown to be involved in Ln-γ1-PrP^C pathway (10).

To investigate the relationship between mGluR5 and PrP^C in the Ln-γ1-induced PrP^C internalization, we treated WT and mGluR5$^{-/-}$ neuronal cultures with Ln-γ1 or scrambled peptide (Ln-SCR) and determined the amount of cell surface PrP^C and mGluR5 by immunofluorescence (Fig. 3, *A–C*) and cell surface biotinylation (Fig. 3, *D–I*). Similar to CF10 cells, in WT neurons, surface localization of PrP^C decreased after treatment with Ln-γ1 peptide for 10 min, but returned to its original level 30 min after the treatment, whereas the scrambled peptide had no effect (Fig. 3, *A*, *B*, *D*, and *F*). In contrast, cell surface PrP^C did not respond to Ln-γ1 peptide treatment in mGluR5$^{-/-}$ neurons (Fig. 3, *A*, *C*, *H*, and *I*). Similarly to what was observed for PrP^C, in WT neurons, surface mGluR5 decreased after 10 min of Ln-γ1 treatment and returned to its original levels after 30 min of treatment (Fig. 3, *E* and *G*). The scrambled peptide had no effect on mGluR5 surface localization. These results suggest that PrP^C and mGluR5 move together upon treatment with the Ln-γ1 peptide. Moreover, the peptide induced internalization of a fraction of cell surface PrP^C (30%), which seems to depend on the complex with mGluR5. It is possible that only the complex PrP^C-mGluR5 is able to respond to Ln-γ1 treatment. As a control for the specificity for the mGluR5 antibody, we detected no mGluR5 signal in mGlur5$^{-/-}$ protein extracts (supplemental Fig. S2).

PrP^C and mGluR5 Cooperate to Transduce AβO Signals—The finding that 30% of the cell surface pool of PrP^C is internalized by Ln-γ1 peptides only in the presence of mGluR5 led us to further investigate how this complex modulates the interaction of AβO with neurons. Interestingly, although Ln-γ1 treatment decreases AβO binding to neuronal surfaces, possibly due to PrP^C internalization, this treatment was inefficient against

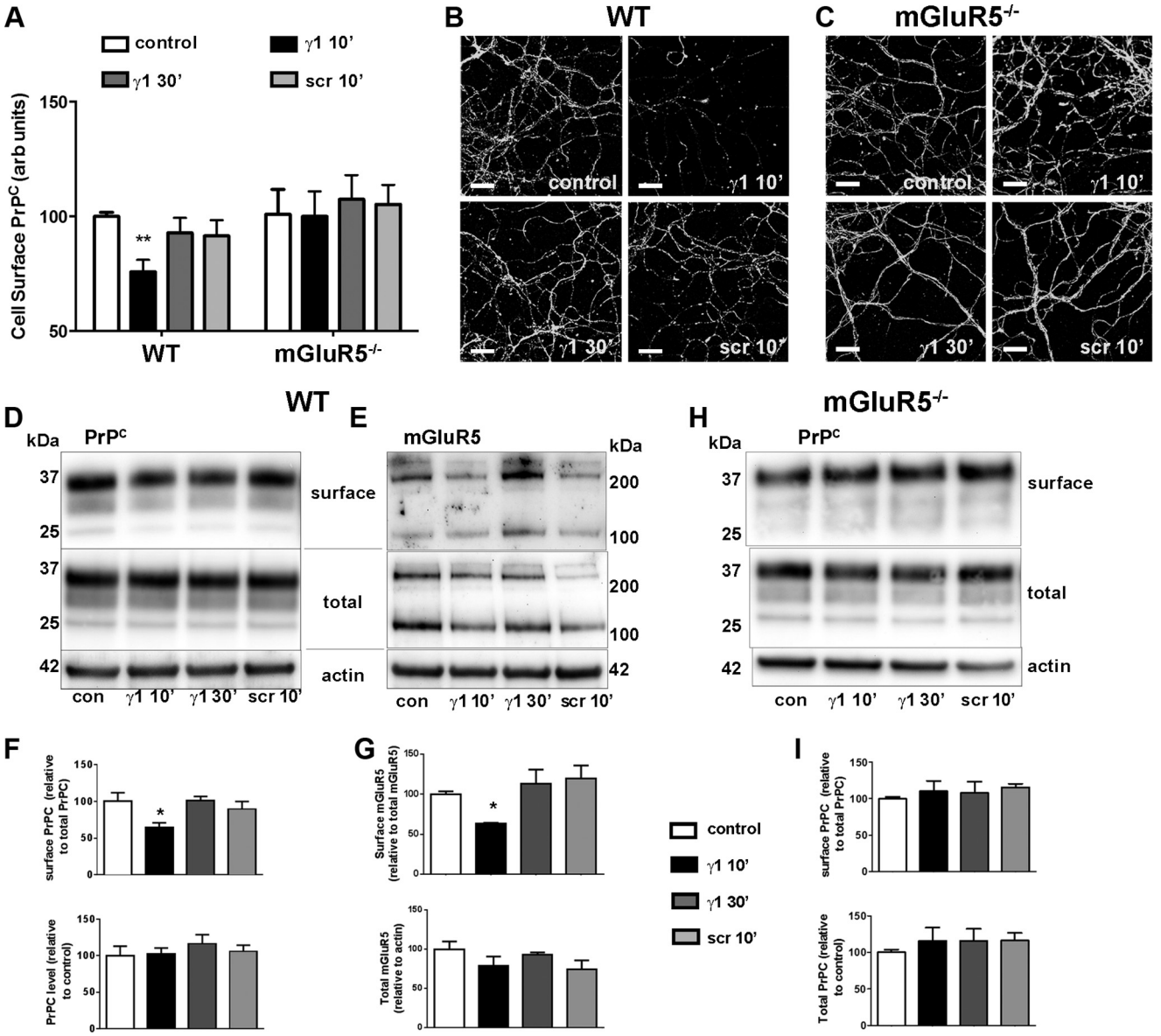

FIGURE 3. **Ln-γ1-induced transient internalization of PrP^C and mGluR5 in primary neurons.** *A–C*, WT (*A* and *B*) and mGluR5$^{-/-}$ (*A* and *C*) hippocampal neuronal cultures were treated with Ln-γ1 for 10 or 30 min, or with Ln-SCR (Ln-γ1 scrambled peptide: IRADIEIKID), and the neuronal cell surface was immunostained with 8H4 antibody. Quantification of surface PrP^C was done for eight random fields of view for each condition (*A*). Primary neuronal cultures were prepared from at least five embryos of each genotype. *arb units*, arbitrary units. *B* and *C*, representative images of WT (*B*) and mGluR5$^{-/-}$ (*C*) cultures, corresponding to each treatment. *Scale bar* = 20 μm. *D–I*, primary cortical neurons were treated with Ln-γ1 for 10 or 30 min, or with Ln-SCR, after which the surface proteins were biotinylated. Levels of surface PrP^C (*D* and *F*) and mGluR5 (monomer + dimer, *E* and *G*) in WT cultures, and of surface PrP^C in mGluR5$^{-/-}$ cultures (*H* and *I*), were quantified as the ratio of biotinylated protein to the total protein levels on Western blot (*D*, *E*, and *H*), and then compared using GraphPad 5.0 software (*F*, *G*, and *I*). *con*, control. *Error bars* indicate mean ± S.E. *, $p < 0.05$.

AβO-induced neuronal death (Fig. 4, *A* and *B*). This result suggests that indeed Ln-γ1 peptide cannot compete effectively with AβOs to prevent toxicity. We then investigated the impact of PrP^C-mGluR5 complex in AβO interaction with neurons. The interaction of AβO with neuronal cell surface was reduced by ~40% in the absence of PrP^C or mGluR5 (Fig. 4, *C* and *D*). In contrast, in neuronal cultures from double knock-out mice lacking both PrP^C and mGluR5, AβO binding to neurites was reduced by 58% (Fig. 4, *C* and *D*). Of note, previous findings in COS-7 cells, overexpressing PrP^C and/or mGluR5, suggested that PrP^C, but not mGluR5, is needed for AβO binding to the cell surface (18). However, Renner *et al.* (39) have shown, similar to the present results, that Aβ binding is decreased in mGluR5-null neurons. To confirm the PrP^C dependence on the effect of Ln-γ1, we performed the same experiments described above in PrP$^{-/-}$ neurons and in CF10 cells. In both cases, in the absence of PrP^C, pretreatment with Ln-γ1 peptide did not alter AβO binding to either PrP$^{-/-}$ neurons or CF10 cells (supplemental Fig. S3). To further explore the role of PrP^C-mGluR5 complex in the toxic effects of AβO, we investigated Ca^{2+} signaling (Fig. 4, *E* and *F*). We confirmed previous results from Um *et al.* (18) that the absence of PrP^C almost abolished Ca^{2+} signaling induced by AβOs. In contrast, some residual Ca^{2+} signaling was still triggered in the absence of mGluR5 (Fig. 4, *E* and *F*). Interestingly, in double-knock-out neurons, lacking both PrP^C and mGluR5, Ca^{2+} signaling was abolished, a sim-

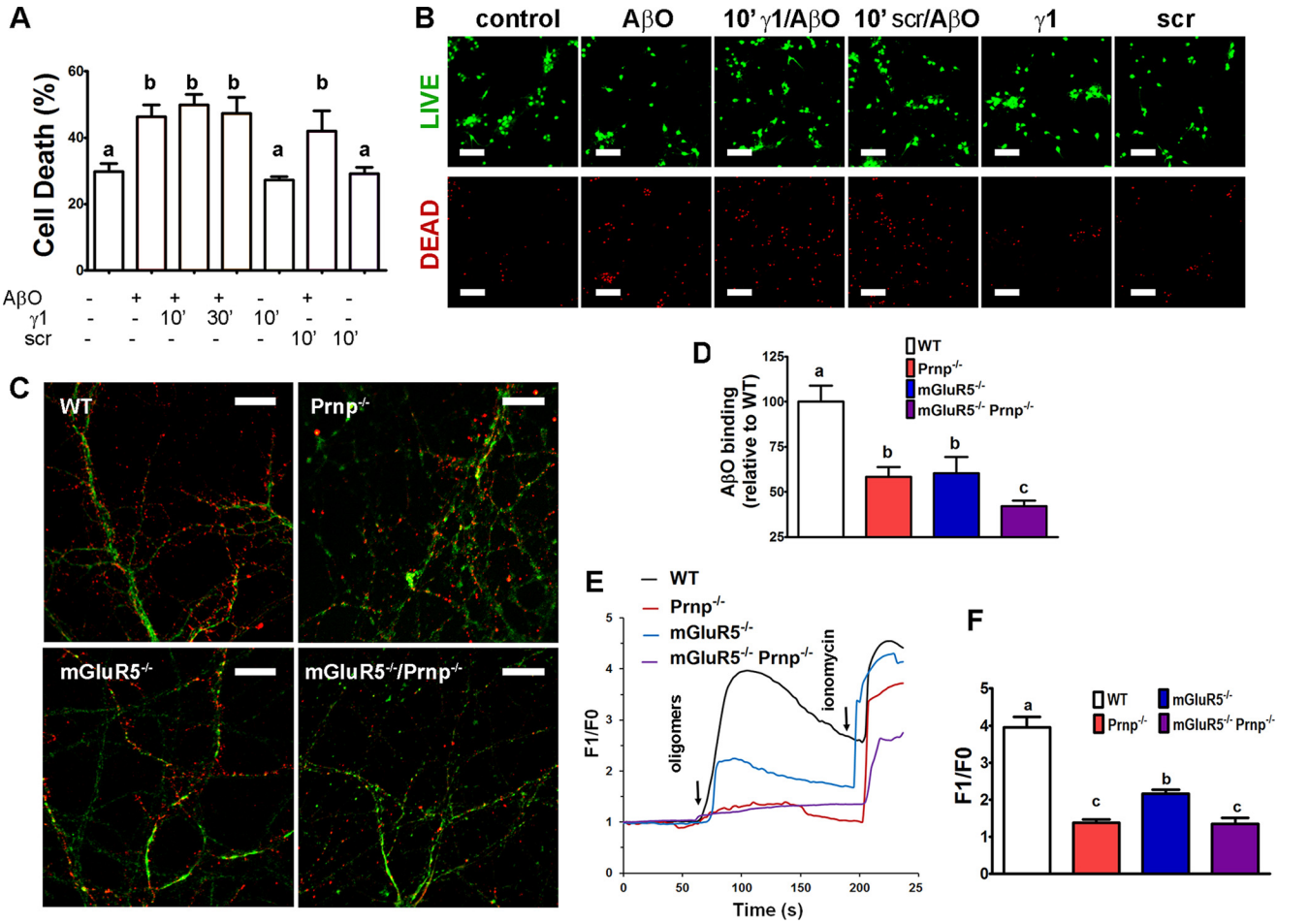

FIGURE 4. **Effect of PrP^C and mGluR5 absence and of Ln-γ1 treatment on AβO toxicity hallmarks.** *A* and *B*, the effect of pretreatment of neuronal cultures with Ln-γ1 or Ln-SCR (*scr*) on AβO-induced cell death. Cell death was registered for cultures prepared from at least five embryos for each condition (*A*). Representative images show live (*green*) and dead (*red*) cells (*B*). *Scale bar* = 100 μm. *C* and *D*, AβO binding to neurites lacking PrP^C, mGluR5, or both proteins. Representative images showing immunostaining of AβOs (*red*) and tubulin (*green*) (*C*). Levels of bound AβOs were normalized to neurite area (calculated by tubulin immunostaining) from eight random fields of view of neuronal cultures prepared from at least five embryos for each genotype (*D*). *Scale bar* = 20 μm. *E* and *F*, AβO-induced increase in intracellular Ca^{2+} in neuron lacking PrP^C (*red*), mGluR5 (*blue*), or both proteins (*purple*). *E*, calcium response (relative to baseline Fluo-4 fluorescence, F_1/F_0) kinetics in the neurons treated with 500 nM AβOs. *F*, quantification of the peak signal from *E*. Data were collected from at least 20 cells in at least 3 independent experiments for each genotype. Statistical comparison was done with one-way analysis of variance followed by Tukey post hoc test. *Error bars* indicate mean ± S.E. For *a* and *b*, $p < 0.05$; for *a* and *c*, $p < 0.001$.

ilar result as observed for neurons lacking only PrP^C (Fig. 4, *E* and *F*).

Prion Disease Onset Is Delayed in mGluR5$^{-/-}$ Mice—Previous studies suggest that various misfolded proteins, including PrP^{Sc}, can interact with and corrupt the signaling mediated by PrP^C, similar to AβOs (44). However, it is unknown whether the PrP^C-mGluR5 complex has a role in other protein misfolding diseases. This might be particularly important in prion diseases, in which PrP^{Sc} toxicity could impact mGluR5 signaling. Therefore, we infected wild-type and mGluR5$^{-/-}$ mice with prions (prion strain RML (named for Rocky Mountain Laboratories)) and followed the mice after infection. Interestingly, there was a significant delay in disease onset, determined by observing clinical symptoms, in mice lacking mGluR5 when compared with control mice (Fig. 5*A*). This, however, did not affect mouse survival (Fig. 5*B*). Biochemical evaluation of protease-resistant PrP^{Sc} and total levels of PrP^C showed no difference between genotypes (Fig. 5, *C* and *D*). These results suggest that pharmacological targeting mGluR5 may delay some prion disease symptoms, but it is unlikely to extend the life of prion disease-affected individuals.

Discussion

In this work, we explored the ensemble PrP^C-mGluR5 and its potential ligands Ln-γ1 and AβOs on the pathological mechanisms involved in neurodegeneration. Our results suggest that Ln-γ1 signals via a small portion of cell surface prion protein. This conclusion is based on the fact that after treatment with a peptide mimicking the effects of Ln-γ1 chain, a transient decrease of 30% in cell surface PrP^C was observed. This minor decrease of cell surface PrP^C prevented subsequent signal by the Ln-γ1 peptide, suggesting that the majority of PrP^C at the membrane is not available for Ln-γ1-induced Ca^{2+} increase. Although the mechanisms involved in this limited signaling by PrP^C-mGluR5 are currently unclear, it is possible that Ln-γ1 peptide may not be able to induce the association of PrP^C with mGluR5, but rather it only activates the complex if it is already formed.

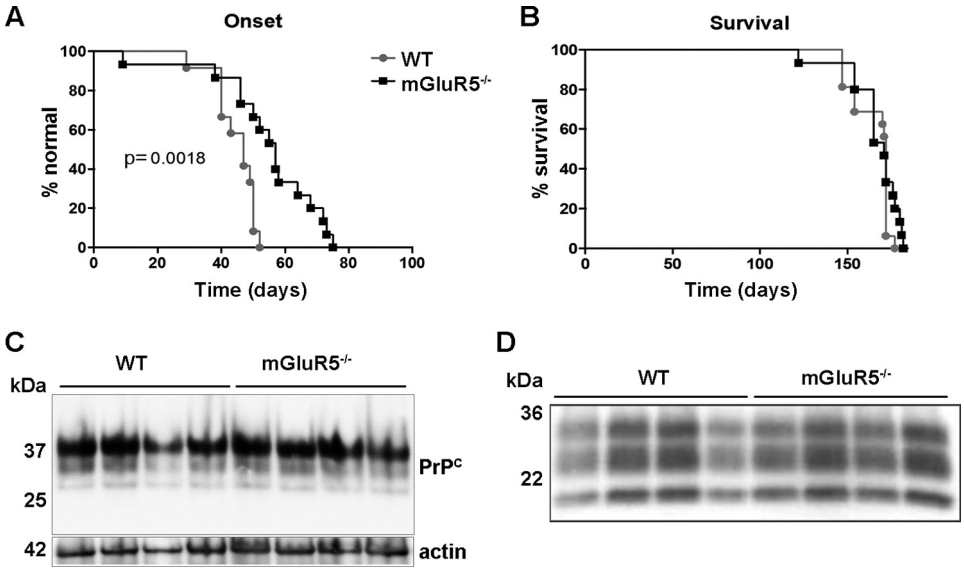

FIGURE 5. **Prion infection in WT and mGluR5$^{-/-}$ mice.** *A*, disease onset in infected mice was determined as described under "Experimental Procedures." *B*, mouse survival upon prion infection. *C*, Western blotting of PrP^C in non-infected WT and mGluR5$^{-/-}$ mice. *D*, Western blotting of PrP^{Sc} in infected WT and mGluR5$^{-/-}$ mice as determined by proteinase K cleavage assay. Four mouse brains were used for each condition for Western blotting analyses. Data were analyzed and compared by Mantel-Cox log-rank test. *A*, $p = 0.0018$, and *B*, $p = 0.3215$.

Evidence that PrP^C can be found in a complex with mGluR5 and mGluR1 without any stimulation is abundant. We (10) and others (18, 19, 25) found that PrP^C and mGluRs can associate biochemically. We have also reported a functional association between PrP^C and mGluR5 in non-neuronal cells for Ln-γ1 peptide signaling (10), whereas others have shown similar functional association for AβOs (18, 25). In the current work, we confirmed and extended these results, in primary neuronal culture from embryos lacking either PrP^C or mGluR5 or both, showing that a complex formed between PrP^C and mGluR5 is important for AβO interaction with neurons. AβO binding to neurons seems to be partially affected by the lack of PrP^C and in the same proportion by the lack of mGluR5. These results are consistent with previous observations that both mGluR5 (10, 18, 25, 39) and PrP^C (21, 23, 27, 33, 38) can function as receptors for extracellular ligands, including AβOs and Ln-γ1. Interestingly, in neurons lacking both mGluR5 and PrP^C, the decrease in AβO binding was not additive. Previous experiments in neurons showed that in mGluR5$^-$ neurons, binding of AβOs is decreased (39), whereas experiments in COS7 cells overexpressing mGluR5 suggests that AβOs cannot bind directly to mGluR5 (18). Overall, our study suggests that although AβO may be able to interact with either PrP^C or mGluR5 independently, as proposed previously (23, 39, 45), a proportion of oligomers may also bind to a preformed complex. These results are consistent with the notion that a certain proportion of PrP^C may already be in a functional complex with mGluR5 at the cell surface.

Trafficking of the GPI-anchored PrP^C has been extensively studied (46–53). Although most GPI-anchored proteins are thought to be internalized via a non-clathrin-mediated mechanism (54), multiple data suggest that PrP^C is internalized in dynamin-sensitive vesicles (48) by clathrin-mediated endocytosis (52, 55–58). Constitutive internalization of cell surface PrP^C seems to depend on LRP1, a scaffold protein that connects PrP^C to clathrin-coated vesicles (59, 60). Here we described a novel mechanism by which Ln-γ1 peptide induces a decrease in only a fraction of cell surface PrP^C, and it can also decrease the same proportion of cell surface mGluR5. Interestingly, the absence of mGluR5 impairs Ln-γ1 peptide-induced internalization of PrP^C, suggesting that the complex PrP^C-mGluR5 may internalize together. mGluR5 can be internalized in a constitutive or an agonist-induced way. Constitutive internalization was found to proceed in a clathrin-independent (61) but rather in a GRK2- (62) and caveolin-1/lipid raft-dependent (63) manner. The mechanisms of agonist-induced endocytosis of mGluR5, which are important for controlling NMDA plasticity in the hippocampus (64), and, possibly, for the roles of mGluR5 in neuropsychiatric disorders (65, 66), are not completely clear. Although PrP^C and mGluR5 have not yet been shown to be present in intracellular vesicles together, both proteins can be found in the post-synaptic density. Moreover, PrP^C co-immunoprecipitates not only with mGluR5 (10, 18), but also with mGluR5 intracellular mediators, including Homer 1b (19), shown previously to regulate mGluR5 trafficking (67). It is possible, therefore, that at least a portion of the PrP^C-mGluR5 complex is internalized together, which could have important implications in mGluR5 functions in healthy organisms and in disease. Of note, AβO treatment of cells can trap both mGluR5 (18) and PrP^C (68) at the cell surface, suggesting that part of the AβO-induced toxic signaling may occur due to the longer permanence of complexes formed between mGluR5 and PrP^C at the cell surface.

It has been suggested that signaling by PrP^C and mGluR5 may be corrupted by AβOs, leading to abnormal activation of Fyn kinase and neurotoxicity (18). Why do different ligands, such as AβO and Ln-γ1, which use similar signaling pathways, have distinct effects? Ln-γ1 interaction with PrP^C triggers neuritogenesis, whereas AβOs cause neurotoxic effects. One possibility is that the ability of these two ligands to trigger internal-

ization or increased permanence of the PrP^C-mGluR5 complex at the membrane could regulate subsequent signaling in neurons. Interestingly, ligands that can trigger internalization of PrP^C, such as Ln-γ1 (present results) or STI1 (46), are usually not toxic and can trigger survival signaling. A second possibility is that Ln-γ1 or its peptide cannot activate the formation of PrP^C-mGluR5 complex, whereas AβOs can (25). These results are consistent with the hypothesis that PrP^C is an extracellular scaffolding protein able to seed the formation of several multiprotein complexes that underlie neuronal signaling (3). Biasing the interaction of PrP^C with other receptors, such as α7 nicotinic acetylcholine receptors (5, 38, 69), may be also an effective way to diminish toxic effects of AβOs.

Despite a critical role for PrP^C and mGluR5 in Alzheimer's disease, it seems that for toxicity, due to PrP^{Sc} buildup, lack of mGluR5 is not critical. Although drugs that interfere with mGluR5 and genetic elimination of mGluR5 seem to improve the outcomes in mouse models of Alzheimer's disease (19, 70), we observed only marginal improvements in clinical signs during prion infection. Our data suggest a small, but unlikely, life-extending benefit, by inhibiting the activity of mGluR5 in prion diseases. It is possible that other mechanisms triggered by the accumulation of PrP^{Sc}, such as endoplasmic reticulum stress (17), which has recently been shown to play a major role in prion and Alzheimer's diseases (71–73), need to be targeted simultaneously with mGluR5 for potential therapeutic benefits in these neurodegenerative diseases.

In summary, we find that Ln-γ1 peptide, which signals via PrP^C-mGluR5, does not affect AβO-mediated neurotoxicity. However, our experiments suggest the possible existence of the complex PrP^C-mGluR5 at the cell surface even in the absence of any ligands. Further experiments are required to determine by which extent the complex PrP^C-mGluR5 can regulate each other's traffic and function in distinct physiological and pathological settings. mGluR5 has been proposed as a target in several psychiatric and developmental conditions, including fragile X syndrome, schizophrenia, and autism spectrum disorders (74–77). It will be important to delineate whether its complex with PrP^C also plays a role in these diseases. Our results support the notion that mGluR5 function is coupled to PrP^C and that targeting this complex might be beneficial in neurodegenerative diseases.

Experimental Procedures

Animals—*Prnp*$^{-/-}$ mice in a C57BL/6 background were kindly donated by Dr. Frank Jirik, University of Calgary (78). mGluR5$^{-/-}$ (Grm5^{tm1Rod}/J) mice in a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, stock number 003558) (79). Procedures were conducted in accordance with approved animal use protocols at the University of Western Ontario (2008/127), University of British Columbia (A11-0138), and the A. C. Camargo Cancer Center (037/09) following Canadian Council of Animal Care (CCAC) and National Institutes of Health guidelines.

Peptide Preparations—AβOs were prepared from Aβ(1–42) peptide (rPeptide) as described previously (38). Briefly, the peptide was monomerized in hexafluoroisopropanol, dried in a SpeedVac centrifuge, restored in DMSO to 1 mM solution, and diluted in PBS (for intracellular calcium experiments) or F-12 medium (Invitrogen) (for the neuronal survival experiments) to a final concentration of 100 μM (hereafter monomer concentration used as AβO concentration). After incubation for 24 h at 4 °C, AβOs were used immediately or stored at −80 °C for no more than 4 weeks. Peptide preparation quality was checked by Western blotting with 6E10 (1:2000, Covance) antibody, and similar preparations were fully characterized using size exclusion chromatography as well as by atomic force microscopy (28, 38). Ln-γ1 (RNIAEIIKDI) or Ln-SCR (IRADIEIKID) peptides were synthesized by GenScript and dissolved in PBS to the final concentration of 1 mM and then used immediately or stored for not more than 2 weeks at −20 °C.

Primary Neuronal Cultures—Mouse primary cortical and hippocampal neuronal cultures were prepared from embryonic day 17 embryos as described previously (10, 38). Briefly, cortices and hippocampi were separated from embryonic brains, dissociated in Hanks' balanced salt solution (Invitrogen), and trypsinized (0.25%) for 20 min at 37 °C). Neurons were plated onto dishes coated with 5 μg/ml poly-L-lysine (Sigma) in minimum essential medium Eagle (Invitrogen) containing 10% FBS (Invitrogen) and antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin). Four hours after plating, the medium was replaced with the Neurobasal medium (Invitrogen), supplemented with B-27 (Invitrogen), glutamine (0.5 mM) (Invitrogen), penicillin (100 units/ml), streptomycin (100 μg/ml), and glucose (0.25%).

Calcium Imaging—Mouse primary hippocampal neurons were plated onto poly-L-lysine-coated 35-mm glass-bottom dishes (MatTek, Ashland, MA) at ~7.5×10^4 cells/dish density. On day 14, cultures were washed with Krebs-Ringer buffer (KRH) (124 mM NaCl, 4 mM KCl, 25 mM HEPES, 1.2 mM $MgSO_4$, and 10 mM glucose), and loaded with 5 μM of the intracellular Ca^{2+} indicator Fluo-4-AM (Invitrogen), diluted in KRH buffer with 2 mM $CaCl_2$, for 1 h at 37 °C. The cells were washed three times with KRH with (for AβO experiments) or without calcium (Ln-γ1 experiments). Data acquisition was performed in a LSM Meta 510 confocal microscope, with excitation at 488 nm (argon laser), and emission was collected with a 505–530-nm band-pass filter. The fluorescence was normalized as F_1/F_0 (F_1, maximal fluorescence after the treatment; F_0, basal fluorescence before the treatment). Imaging analysis was performed using ImageJ software (WCIF ImageJ, National Institutes of Health). Experiments were carried out with ≥3 different dishes, and 20–30 cells were monitored in each experiment.

CF10 Cell Culture and Ca^{2+} Imaging—CF10, a PrP^C-null immortalized cell line, and its counterpart expressing 3F4-tagged mouse PrP^C were obtained as described previously (46). CF10 cells were plated onto 35-mm glass-bottom dishes (MatTek) (10^4 cells), and then serum-starved for 48 h with medium change after 24 h. Cells were loaded with Fluo-4-AM (5 μM/30 min) and then washed three times with KRH buffer. Data acquisition was performed in a confocal Bio-Rad Radiance 2100/Nikon TE2000U microscope, with excitation at 488 nm (argon laser), and emission was collected with a 522–535-nm band-pass filter as described previously (10).

CF10 Cell Surface Protein Biotinylation and Western Blotting—Biotinylation of cell surface proteins was performed as described (46, 80). Briefly, cells were incubated with Ln-γ1 (120 μM) for 10 or 30 min and then transferred to ice, washed, and incubated on ice in PBS/CM (PBS supplemented with 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$). Cell surface proteins were biotinylated with sulfo-NHS-SS-biotin (Pierce) for 40 min on ice. To quench the biotinylation reaction, cells were washed and incubated for 30 min with cold 100 mM glycine in PBS/CM, followed by three washes with cold PBS/CM, and then proteins were extracted using 100 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholic acid, pH 7.4. Biotinylated proteins were separated from non-biotinylated proteins by pulldown of NeutrAvidin beads from equivalent amounts of total cellular protein (800 μg) from each sample. The biotinylated proteins were subjected to SDS-PAGE, followed by electroblotting onto PVDF membrane, and then revealed using a mouse anti-PrP^C antibody as described previously (81). For quantification, the major glycosylated band of PrP^C in non-saturated blots was analyzed using ImageQuant TL and normalized by the expression of PrP^C in the lysates.

Neuronal cell Surface Protein Biotinylation and Western Blotting—Mouse primary cortical neurons were plated on poly-lysine-coated 60-mm Petri dishes at ~3×10^6 cells/dish density. On day 8, cultures were treated with Ln-γ1 or Ln-SCR for a specific amount of time after which the cells where washed with ice-cold PBS and the cell surface was biotinylated using the cell surface protein isolation kit (catalogue number 21328, Thermo Scientific) as described in the supplier's manual. In short, cells were incubated with NHS-SS-biotin reagent for 1 h, washed, and lysed. Biotinylated protein was collected from the lysate by incubation with NeutrAvidin agarose, followed by elution with 50 mM dithiothreitol. Untreated lysate was used for total protein quantification. PrP^C and mGluR5 in cell surface or total cellular fractions were quantified by Western blotting using 8H4 (1:2500, Abcam ab61409, Lot: GR82819-6) and anti-mGluR5 (1:1000, Millipore AB5675, Lot: 2279534) antibodies, respectively.

Peptide Binding and Protein Internalization Imaging—For AβO binding and PrP^C internalization experiments, primary neuronal cultures were plated on glass coverslips at ~5×10^4 cells/dish density. On day 15, cultures were washed with KRH buffer and treated with 250 nM AβO solution in KRH buffer for 15 min or with 40 μM Ln-γ1 or Ln-SCR solution for 10 or 30 min. After that, cover glasses were washed with ice-cold PBS, permeabilized for 5 min in PBS containing 0.5% Triton X-100, and fixed with 4% paraformaldehyde for 20 min. For immunofluorescence, cover glasses were incubated with 6E10 (for AβO binding, 1:350, Covance, catalogue number SIG-39300, lot number D13EF01399) or 8H4 (for PrP^C internalization, 1:350) and anti-tubulin (1:300, Abcam) antibodies overnight at 4 °C, followed by Alexa Fluor 633 anti-mouse IgG and Alexa Fluor 488 anti-rabbit IgG (1:1000, Invitrogen) for 1 h at room temperature. After that, cover glasses were mounted on slides using Immu-mount (Thermo Fisher Scientific) and imaged with an LSM 510 Meta ConfoCor microscope. Integrated fluorescence intensity was normalized by total neurite area calculated from tubulin fluorescence, and then for each treatment normalized toward the wild-type or non-treated controls.

Surface Plasmon Resonance—SPR was measured using a Biacore X system (GE Healthcare Life Sciences) equipped with a nitrilotriacetic acid sensor chip as described previously (38). Briefly, the chip was nickel-charged and uniformly covered with His_6-tagged PrP to the final SPR signal of ~10,000 response units. Ln-γ1, AβO, and their mix were diluted in 25 mM HEPES, 150 mM NaCl, 10 mM imidazole, pH 7.0, and injected at 5 μl/min. On-kinetics were registered for 6 min, followed by off-kinetics registered for 2 min. The chip surface was regenerated between injections by a short injection of 10 mM HCl. SPR curves for binding to PrP were analyzed by maximum signal comparison.

LIVE/DEAD Cell Viability Assay—Mouse primary hippocampal neurons were plated onto poly-L-lysine-coated 4-well dishes and cultured for 10 days. On day 11, AβOs (1 μM) were added to cultures. Some cultures were pretreated with Ln-γ1 or Ln-SCR peptides (40 μM) for 10 or 30 min. Following 48-h treatment, cultures were analyzed with a LIVE/DEAD cell viability assay (Invitrogen) according to the manufacturer's manual. Live and dead cells were counted from fluorescence images taken with Zeiss LSM 510 Meta confocal microscope equipped with 10×/0.3 objective lens, and the cell death rate was calculated as the percentage of dead cells in the total amount of cells.

Prion Infection and Western Blotting—For prion infection the mice were anesthetized with isoflurane. The injection was made through the skull into the brain with a 26-gauge needle attached to a syringe containing the prion material (5% RML strain). The total volume injected per mouse was 20 μl. Following the injection, the mice were allowed to recover from the anesthesia in their home cage. Early signs of mouse prion disease were evaluated including ataxia (lack of coordination) and extension of the hind limbs when mice are hung by their tails. As the disease progressed, affected mice showed hypokinesia, waddling gait, difficulty righting from a supine position, weight loss, and deficient grooming. When held by their tails, some affected mice assume an unusual flexed posture, with all four limbs clasped together. For the onset of the disease, the following parameters were analyzed: 1) foot clasp reflex when picked up by the tail and 2) observation of kyphosis while the mouse was in the cage. These clinical observations were done on a weekly basis prior to onset and daily upon onset of disease. Experimental end point was reached once a mouse no longer could right itself within 30 s. The incubation period when injecting 20 μl of 5% RML strain intracranially is ~120 days. Once the mice reached the experimental end point (described above), they were euthanized by inhalation of CO_2 and their brains were harvested and frozen on dry ice.

For prion scrapie analyses, brain homogenate in PBS (250 μg of total protein) was digested with 50 μg/ml proteinase K for 1 h at 37 °C, and then samples were boiled in SDS loading buffer and separated with 14% SDS-PAGE. After electrotransfer to a PVDF membrane (Immobilon, Millipore) protein bands were probed with SHA31 antibody (1:30000, Spibio Inc., catalogue number A03263, lot number 0107).

Statistics—Western blots and immunocytochemistry data were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD) and statistically analyzed using one-way analysis of variance with Tukey's post hoc or by Student's *t* test using Prism software (GraphPad, La Jolla, CA). For prion disease onset and progression, data were analyzed and compared by Mantel-Cox log-rank test.

Author Contributions—F. H. B. and V. G. O. conceived and designed the project, acquired data, analyzed and interpreted data, and wrote the manuscript; F. A. C., A. L. S. G., G. D. S. F., N. D., L. B., and K. O. P. C. N. acquired data; J. L. S., D. W., N. R. C., and V. F. P. analyzed and interpreted data; and V. R. M. and M. A. M. P. conceived and designed the project, analyzed and interpreted data, and wrote the manuscript.

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