Involvement of PrP\textsuperscript{C} in kainate-induced excitotoxicity in several mouse strains

Patricia Carulla\textsuperscript{1,2,3}, Franc Llorens\textsuperscript{1,2,3,4}, Andreu Matamoros-Angles\textsuperscript{1,2,3}, Patricia Aguilar-Calvo\textsuperscript{5}, Juan Carlos Espinosa\textsuperscript{5}, Rosalina Gavín\textsuperscript{1,2,3}, Isidre Ferrer\textsuperscript{6,7}, Giuseppe Legname\textsuperscript{8}, Juan Maria Torres\textsuperscript{5} & José A. del Río\textsuperscript{1,2,3}

The cellular prion protein (PrP\textsuperscript{C}) has been associated with a plethora of cellular functions ranging from cell cycle to neuroprotection. Mice lacking PrP\textsuperscript{C} show an increased susceptibility to epileptic seizures; the protein, then, is neuroprotective. However, lack of experimental reproducibility has led to considering the possibility that other factors besides PrP\textsuperscript{C} deletion, such as the genetic background of mice or the presence of so-called “Prnp flanking genes”, might contribute to the reported susceptibility. Here, we performed a comparative analysis of seizure-susceptibility using characterized Prnp\textsuperscript{+/+} and Prnp\textsuperscript{0/0} mice of B6129, B6.129, 129/Ola or FVB/N genetic backgrounds. Our study indicates that PrP\textsuperscript{C} plays a role in neuroprotection in KA-treated cells and mice. For this function, PrP\textsuperscript{C} should contain the aa32–93 region and needs to be linked to the membrane. In addition, some unidentified “Prnp-flanking genes” play a role parallel to PrP\textsuperscript{C} in the KA-mediated responses in B6129 and B6.129 Prnp\textsuperscript{0/0} mice.

Although the role of the cellular form of the prion protein (PrP\textsuperscript{C}) in living organisms has been intensively studied, a clear consensus concerning the physiological functions of this protein is still elusive and controversial. To date, existing evidence implicates PrP\textsuperscript{C} in numerous distinct cellular processes, including cell proliferation and differentiation\textsuperscript{1,2}, copper homeostasis\textsuperscript{3,4}, oxidative stress\textsuperscript{5} and cell signaling\textsuperscript{6,7}, among others.

The generation of different transgenic Prnp\textsuperscript{0/0} mice (mixed B6129 Prnp\textsuperscript{Zrchl/Zrchl} or co-isogenic 129/ Ola Prnp\textsuperscript{Edbg/Edbg} backgrounds) in the early 1990s did not reveal any relevant phenotypic alteration of the mutant mice\textsuperscript{8,9}. However, subsequent studies identified an abundance of phenotypic alterations (e.g.,\textsuperscript{10}), including depressive-like behaviour\textsuperscript{11}, cognitive deficits\textsuperscript{12}, age-dependent behavioural abnormalities\textsuperscript{13}, altered olfaction\textsuperscript{14}, peripheral myelin deficits\textsuperscript{15}, altered circadian rhythms\textsuperscript{16} and an increased susceptibility to oxidative stress\textsuperscript{5} and glutamate excitotoxicity\textsuperscript{17–19}. Indeed, different laboratories have described in these strains and in congenic B6.129 Prnp\textsuperscript{Zrchl/Zrchl} (B6129 mice backcrossed with C57BL/6 for several generations) an enhanced sensitivity to seizures after the administration of epileptogenic drugs such as kainic acid (KA), N-methyl-d-aspartic acid (NMDA), pilocarpine and pentylenetetrazol (PTZ), suggesting a neuroprotective role of the protein against excitotoxic insults (e.g.,\textsuperscript{17–21}). However, some studies suggest that PrP\textsuperscript{C} is not involved in KA-mediated excitotoxicity and that the observed differences are likely

\textsuperscript{1}Molecular and Cellular Neurobiotechnology, Institute of Bioengineering of Catalonia (IBEC), Parc Científic de Barcelona, Barcelona, Spain. \textsuperscript{2}Department of Cell Biology, Universitat de Barcelona, Barcelona, Spain. \textsuperscript{3}Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Barcelona, Spain. \textsuperscript{4}German Center for Neurodegenerative Diseases (DZNE), Robert-Koch Str. 40, 37075, Göttingen, Germany. \textsuperscript{5}Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, Spain. \textsuperscript{6}Institut de Neuropatologia, IDIBELL-Hospital Universitari de Bellvitge, Hospitalet de Llobregat, Barcelona, Spain. \textsuperscript{7}Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED) Barcelona, Spain. \textsuperscript{8}Laboratory of Prion Biology, Department of Neuroscience, Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste, Italy. Correspondence and requests for materials should be addressed to J.A.D.R. (email: jadelrio@ibecbarcelona.eu)
to be associated with the genetic background of the mice used in the experiments. In fact, it was described some years ago how different mouse strains exhibit different vulnerability to the administration of glutamate and other seizure-inducing drugs, which correlates with differences in axonal sprouting and cell death in the hippocampal region (e.g., ). For example, the hippocampus of the C57BL/6 background mouse, in contrast to FVB/N, is more resistant to the excitotoxic effects of KA. Thus, these genetic differences dilute the specific participation of PrP in KA-mediated cell death. However, these genic influences do not per se explain the neuroprotective properties of PrP observed in KA-treated neuroblastoma cell lines carrying different dosages of the Prnp gene (see below).

In addition to these genetic differences, a number of Prnp-flanking genes associated with the 129 genotype in B6.129 Prnp/Zrchl/Zrchl mixed mice have recently been described. These genes are retained in Prnp progeny of congenic B6.129 Prnp/Zrchl/Zrchl after numerous (>10) crosses with C57BL/6 mice. SNP analysis of the backcrossed mice indicated that after 5 to 6 rounds the amount of C57BL/6-associated SNP increased from 60 up to 93%. Thus, B6.129 Prnp/Zrchl/Zrchl wild type and mutant mice may still differ at these additional polymorphic loci associated with Prnp. One of these loci, the signal regulatory protein alpha (SIRPs) has recently been described as responsible for a previously PrP-associated phagocytic function in macrophages. More relevantly, overexpressing mice (Tg20) generated in mixed B6.129 Prnp/Zrchl/Zrchl congenic mouse carried several copies of the polymorphic loci, since in most cases they are also crossed with previously backcrossed B6.129 Prnp/Zrchl/Zrchl mice. In this scenario, it is reasonable to consider that the data obtained using Prnp or Tg20 (backcrossed or not with C57BL/6) in electrophysiological studies may yield conflicting results. Indeed, lack of PrP has been associated with long-term potentiation (LTP), GABA-mediated fast after hyperpolarization (AHP) and paired pulse facilitation (PPF) abnormalities in mutant mice (e.g., ). However, regulatory participation of PrP in neurotransmission and neuroprotection and in other cellular functions has also been shown with acute modulation of Prnp expression in neural cell lines and in other organisms (e.g., zebrafish) as models, to avoid the above-mentioned genetic problems of mice. For example, neuroblastoma (N2a) cells with reduced Prnp expression are more susceptible to KA in contrast to non-modified cells.

Functions of PrP in neurotransmission are also reinforced since i) PrP seems to be located in the synaptic cleft; ii) several synaptic proteins (e.g., synaptophysin, synapsin I, II, GluR6/7, NR2D, GluR1/2, mGluR1/5) and ion channels have been identified as interacting partners of PrP; iii) PrP regulates GluR6/7-mediated signalling through the modulation of the formation of the GluR6/7-PSD-95-MLK3 trimeric complex, which triggers the activation of the JNK3 apoptotic pathway in the hippocampus in response to KA administration; and iv) it modulates the NMDA receptor.

Due to these conflicting results, we aimed to clarify the participation of PrP in KA-mediated excitotoxicity in vivo, using B6.129 Prnp/Zrchl/Zrchl, B6.129 Prnp/Zrchl/Zrchl, 129/Ola Prnp/Edbg/Edbg and FVB Prnp/0/0 and Prnp/+ mice, and in vitro using N2a cells. In addition, we wished to explore whether the expression of truncated forms of PrP (ΔF35 or ΔC4) lacking 32–134 and 32–93 residues of PrP might increase the neurotoxic effects.

Our results indicate that FVB/N Prnp mice are not suitable for the in vivo model of KA-mediated excitotoxicity due to the intrinsic sensitivity to KA of FVB/N mice that hides clear differences. More relevantly, both B6.129 Prnp/Zrchl/Zrchl and co-isogenic 129/Ola Prnp/Edbg/Edbg mice display more severe epileptic episodes and neurotoxic brain damage than their corresponding wild-type controls. Backcrossed B6.129 Prnp/Zrchl/Zrchl mice (with 93% of C57BL/6 SNPs) displayed lower numbers of seizures compared to B6.129, indicating additional participation of Prnp-linked genes in KA-mediated effects in B6.129 Prnp/Zrchl/Zrchl mice. However, our data using co-isogenic 129/Ola Prnp/Edbg/Edbg (100% 129 genotype) and cell lines reinforce the notion that PrP is neuroprotective in KA treatment. In addition, the expression of ΔF35 and ΔC4 in a B6.129 Prnp/Zrchl/Zrchl background increased hippocampal cell death, especially in the CA3 region after KA injections. These in vivo data were also corroborated using N2a cells transiently expressing N-terminal truncated forms of PrP. Lastly, our in vitro experiments also suggest that to be neuroprotective, PrP should be bound to the plasma membrane by means of the GPI moiety.

**Results**

**KA-induced seizures in several strains of wild-type and PrP-null mice.** A gene-targeting strategy to generate null mutations in mice is a powerful research tool to reveal in vivo the function of a single protein, as derived phenotypic alterations are usually attributed to the deleted gene. Nevertheless, behavioural alterations observed in null-mutant mice could result in some cases from the genetic background (see introduction, see also ). In order to determine a possible influence of non-Prnp genes in the susceptibility to KA-induced seizures, we compared the epileptic response in B6129 (n = 20), 129/Ola Prnp/Edbg/Edbg (n = 9) and FVB/N Prnp/0/0 (n = 7) and wild type mice (B6129, n = 16; 129/Ola Prnp/Edbg/Edbg (n = 11); FVB/N (n = 8) (Fig. 1a and Supplementary Tables 1–6). A multiple administration protocol, consisting of three intraperitoneal injections of KA (10 mg/kg body weight) at 30 min intervals, was used in this experiment. Seizure intensity was analysed during the 4 hours after the first KA injection and scored as indicated in Methods. For an easier comparative analysis, data from grades I to IV were grouped together.

After the behavioural study, mice were numbered and kept in separate boxes until histological studies. Percentages of the different strains of mice reaching each stage were represented (Fig. 1a). As indicated
Figure 1. Comparison of KA-induced seizure profile in Prnp<sup>+/+</sup> and Prnp<sup>0/0</sup> mice on B6129, B6.129, 129/ Ola or FVB/N genetic backgrounds. (a) Percentage of mice reaching seizure stages I to VI and KA-induced mortality. Adult animals were subjected to a multiple KA-injection protocol (10 mg/kg b.w.) and epileptic responses were analysed during 4 hours after the first KA injection (see Methods for details). (b) Example of the western blot detection of PrP<sup>C</sup> (6H4) expression in brain extracts obtained from B6129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>+/+</sup>, B6129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>0/0</sup>, 129/Ola Prnp<sup>Edbg/Edbg</sup> Prnp<sup>+/+</sup>, 129/Ola Prnp<sup>Edbg/Edbg</sup> Prnp<sup>0/0</sup>, FVB/N Prnp<sup>+/+</sup> and FVB/N Prnp<sup>0/0</sup> mice. Tubulin was used as a loading control. (c) Photomicrographs showing the pattern of neurodegeneration (Fluoro-Jade B staining) and DAPI staining of hippocampus in B6129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>+/+</sup>, B6129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>0/0</sup>, 129/Ola Prnp<sup>Edbg/Edbg</sup> Prnp<sup>+/+</sup>, 129/Ola Prnp<sup>Edbg/Edbg</sup> Prnp<sup>0/0</sup>, 24 hours after KA treatment. Dying cells are mainly located in the pyramidal cell layer of CA1 and CA3 regions of B6129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>+/+</sup> and 129/Ola Prnp<sup>Edbg/Edbg</sup> Prnp<sup>0/0</sup> mice respectively. (d) Time course of stage VI seizure during the first 30 min, from 30–60 min and from 60–180 minutes, of adult B6129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>0/0</sup> vs B6.129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>0/0</sup>. Data are presented as the mean ± S.E.M. of the number of seizures/number of animals. Note the lower levels of seizures in parallel with decreased 129 associated genes in B6.129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>0/0</sup> mice. Abbreviations: sr, stratum radiatum; sl, stratum lucidum; slm, stratum lacunosum-moleculare; sp, stratum pyramidale; so, stratum oriens. Scale bar represents 200 μm. Asterisks indicate statistical significance (**P < 0.01, Mann-Whitney U test).
(Fig. 1a and Supplementary Tables 1–6), all mice achieved stages I–IV, developing hypoactivity and immobility shortly after the first injection. When comparing wild type vs Prnp-null mice, significant differences were observed in the percentage of mice reaching stages V and VI, with the B6129 PrnpZrchl/Zrchl and 129/Ola PrnpZrchl/Zrchl mice much more susceptible to seizures than their respective Prnp+/+ controls (Fig. 1a and Supplementary Movies 1 and 2). In both groups, a high percentage of Prnp-null mice (100% and 75% respectively) developed loss of balance control and intermittent whole-body convulsions. More severe seizure activity, consisting of continuous seizures and or ‘popcorn bouncing’ behaviour (stage VI) was also reported, leading to the death of 20% of the B6129 PrnpZrchl/Zrchl mice tested. In contrast, no B6129 Prnp+/+ or 129/Ola Prnp+/+ mice reached stage VI or died during experiments.

Distinct results were obtained when analysing FVB/N mice, since no significant differences were observed between FVB/N Prnp+/+ and FVB/N Prnp0/0 animals, and only a 9% difference was observed between them (Fig. 1a and Supplementary Movie 3). FVB/N Prnp+/+ showed a high epileptic response at 10 mg/kg b.w of KA (≈50%), which was not observed in B6129 Prnp+/+ (≈30%) and 129/Ola Prnp+/+ (0%); it occurred in all described behavioural stages, but without any reported deaths. Although other unknown factors might be involved, such phenotypic differences between Prnp+/+ mouse strains could be related to small differences in PrPC expression levels, since our western blot analysis revealed lower PrPC expression in FVB/N Prnp+/+ (0.62 ± 0.04) compared to 129/Ola Prnp+/+ (0.92 ± 0.038) mice (t = 4.21, mean diff. = 0.30; 95% CI of diff. = 0.66 to 0.54) but not between B6129 Prnp+/+ (0.84 ± 0.63) and FVB/N Prnp+/+ mice (t = 3.104, mean diff. = 0.22; 95% CI of diff. = 0.03 to 0.459), or between B6129 Prnp+/+ and 129/Ola Prnp+/+ (t = 1.112, mean diff. = 0.08; 95% CI of diff. = -0.3166 to 0.1566) (Fig. 1b and Supplementary Fig. 1). It has been previously reported that excitotoxic damage derived from KA administration primarily affects pyramidal cells from CA1 and CA3 regions of the hippocampus. To corroborate that the increased cell death is associated with PrPc deletion, we focused on B6129 Prnp0/0 and 129/Ola Prnp0/0 mice. We obtained coronal brain sections (dorsal hippocampus) from these mice 24 hours after KA treatment and performed Fluoro-Jade B staining to determine the associated neurodegeneration in the hippocampus. Wild type mice were processed in parallel (Fig. 1c). B6129- Prnp0/0 mice displayed much more pronounced pyramidal cell damage, with higher numbers in CA1 than in CA3, than wild-type controls, in which no Fluoro-Jade B positive cells were observed. PrPc-dependent differences were also detected between 129/Ola- Prnp0/0 and 129/Ola-Prnp+/+ although cell death appeared to be restricted mostly to the CA3 region (Fig. 1c).

Taken together, the present results support the notion that the genetic background of the different Prnp0/0 mice plays a role in KA susceptibility 22,23, with the FVB/N background more prone to seizures than 129/Ola or the mixed genetic background B612926,49. The susceptibility of the FVB background 49,50 probably masks participation of PrPc at this specific KA-dosage.

More relevantly, the present results demonstrate a role of PrPc in neuroprotection, since the phenotypic differences observed between Prnp0/0 and Prnp+/+ mice in B6129 PrnpZrchl/Zrchl and 129/Ola PrnpZrchl/Zrchl mice generated after crossing the original B6129 PrnpZrchl/Zrchl mice with C57BL/6 mice over several generations (8–10) (129 in origin). We backcrossed these B6129 PrnpZrchl/Zrchl mice to C57BL/6 mice to reduce the non-C57BL/6 microsatellite markers to ≈30% (Fig. 1d and Supplementary Movie 1). In addition, the differences between the numbers of seizures can be restricted mostly to the CA3 region (Fig. 1c).

Decreased seizures in B6.129 PrnpZrchl/Zrchl compared to B6.129 PrnpZrchl/Zrchl mice. In a second set of experiments we used B6.129 PrnpZrchl/Zrchl mice generated after crossing the original B6129 PrnpZrchl/Zrchl mice with C57BL/6 mice over several generations. In these experiments, B6129 PrnpZrchl/Zrchl mice were purchased directly from EMMA (Monterotondo, Italy) and they carried approximately 64% of C57BL/6 microsatellite markers (Charles River Laboratories) with 46% of non-C57BL/6 markers (129 in origin). We backcrossed these Prnp0/0 mice with C57BL/6 mice over several generations (8–10) to reduce the non-C57BL/6 microsatellite markers to ≈6.5–7%. The presence of C57BL/6 or 129 marker alleles in all phenotypes used in the present study was determined by the genetic testing service at Charles River Laboratories. In the test, 110 microsatellite markers at approximately 15 cm intervals were analyzed, spreading across the 19 autosomes and the X chromosome, which distinguishes among 129 microsatellite markers ranging from 92 to 94% of C57BL/6 (in B6.129 PrnpZrchl/Zrchl mice). Thus B6129 and B6.129 PrnpZrchl/Zrchl mice were treated with 8 mg/kg b.w. (B6129 n = 8 and B6.129 PrnpZrchl/Zrchl n = 6) or 10 mg/kg b.w (B6129 n = 8 and B6.129 PrnpZrchl/Zrchl n = 9) of KA and the number of seizures was determined during the first 30 minutes, from 30 to 60 minutes and from 60 to 180 minutes postinjection. In this experiment each mouse received a KA injection every 30 minutes as above. Data revealed a relevant number of seizures in B6129 PrnpZrchl/Zrchl Prnp0/0 mice compared with B6.129 PrnpZrchl/Zrchl Prnp0/0 mice (KA 8 mg/kg P = 0.0021; KA 10 mg/kg P = 0.0086; Mann-Whitney U test confidence interval (CI) = 95% (Fig. 1d and Supplementary Movie 1). In addition, the differences between the numbers of seizures can be observed in both KA concentrations (8 and 10 mg/kg b.w). These data indicate that although PrPc plays a role in the increased susceptibility to KA as demonstrated above, the 129 associated genes also play a role in the observed results.

Increased susceptibility in B6.129-Prnp0/0 and 129/Ola- Prnp0/0 correlates with enhanced cell death, inflammatory markers and astrogliosis. Parallel histological sections to those processed in Fig. 1 KA-induced astrocitic activation and inflammatory response in B6129-Prnp0/0 (n = 5), 129/Ola-Prnp0/0 (n = 5) mice at their respective controls (n = 5 each genotype) (Fig. 2a). Immunohistochemical analysis of GFAP-positive cells in the hippocampus of PrPc-null mice (either B6129 or 129/Ola) revealed large numbers of positive cells in the hippocampal CA1-3 when compared to Prnp+/- controls.
sections of B6129 stratum radiatum Prnp details). Decreased levels of PrPC after treatments were detected with western blot (pcDNA-Mann-Whitney mice (Tg44 chemical elimination of the GPI domain and of PrPC reduces neuroprotection to KA in Figure 2. (a) Examples of GFAP-positive immunoreactive cells in the stratum radiatum of the hippocampal CA1-3, 24 hours after KA administration in hippocampal coronal sections of B6129 Prnpzx/zch/Prnp+/+, B6129 Prnpzx/zch/Prnp0/0, 129/Ola Prnpdxg/Edg Pnpr++, 129/Ola Prnpdxg/Edg Pnpr0/0. Both Prnp-null mice show greater numbers of reactive astrogia than their wild-type controls. (b) RT-qPCR of TNFα and IL1β mRNA levels from hippocampal RNA extracts obtained from B6129 Prnpzx/zch/Prnp+/+, B6129 Prnpzx/zch/Prnp0/0, 129/Ola Prnpdxg/Edg Pnpr+/+, 129/Ola Prnpdxg/Edg Pnpr0/0 mice 6 hours after the last KA treatment (at 60 minutes). Plotted data (mean ± S.E.M.) were obtained from three independent experiments and represented as mean fold change induction. Abbreviations are as in Fig. 1. Scale bars represent 100 μm. Asterisks indicate statistical significance (**P < 0.01 Mann-Whitney U test).

(B6129-Prnp0/0 75.7 ± 1.24 vs B6129-Prnp+/+ 43.4 ± 1.06; P = 0.005 Mann-Whitney U test. 129/Ola-Prnp0/0 53.7 ± 1.44 vs 129/Ola-Prnp+/+ 42.9 ± 0.92; P = 0.0047 Mann-Whitney U test. Immune-reactive cells showed hypertrophic cell bodies and thicker glial processes (Fig. 2a). Moreover, quantitative real-time PCR data obtained from hippocampal samples 6 hours after KA-administration showed upregulation of the main pro-inflammatory markers TNFα (4.7-fold increase in B6129-Prnp0/0 (n = 3) P = 0.0046, Mann-Whitney U test and 3.8-fold increase in 129/Ola-Prnp0/0 (n = 3) (P = 0.0008, Mann-Whitney U test) vs respective wild type (B6129 n = 3 and 129/Ola n = 3) and IL1β (3.7-fold increase in B6129-Prnp0/0 and 3.0-fold increase in 129/Ola-Prnp0/0 vs respective wild type (B6129- Prnp0/0 P = 0.0046 and 129/Ola Prnp0/0 P = 0.0004, and Mann-Whitney U test)) (Fig. 2b).

Chemical elimination of the GPI domain and of PrP C reduces neuroprotection to KA in Prnp-transfected N2a cells. The GPI anchor in PrP C, as in other GPI-proteins, is not only necessary for the stability and attachment of the protein to the cell surface, but also for its association to specialized membrane microdomains (e.g., lipid rafts), its intracellular traffic and signal transduction events. In addition, the GPI group has been suggested as playing a role in prion disease toxicity, as transgenic mice expressing secreted forms of PrP C lacking its GPI-moiety showed no clinical symptoms despite accumulating PrP Sc in plaques 51. Because in our microsatellite analysis the C57BL/10-PrP GPIless mice (Tg44+/+ kindly provided by Dr. B. Chesebro) contained non-B10 regions in chromosome 3 and in chromosome 2 flanking the Prnp locus, we decided to check whether the degradation of the GPI binding domain of the PrP C leading to a decreased PrP C in the plasma membrane could overcome the neuroprotective function of PrP C (Fig. 3a–c). In addition, this approach would determine whether the neuroprotective effect against KA treatment of PrP C takes place at the membrane or intracellularly. Thus, N2a cells were treated either with Phospholipase C (PLC) enzyme or Glimepiride, a sulphonylurea approved for the treatment of diabetes mellitus, inducing the release of PrP C from the surface of prion-infected neuronal cells, which releases PrP C from the surface of neuronal cells 52 (see methods for details). Decreased levels of PrP C after treatments were detected with western blot (pcDNA = 0.39 ± 0.021; pcDNA-PrPC = 0.91 ± 0.025; pcDNA-PrPC + PLC = 0.45 ± 0.013; pcDNA-PrPC + Glia = 0.496 ± 0.014) (Fig. 3a and Supplementary Fig. 2) or with immunocytochemistry in non-permeabilized cells (Fig. 3b) (pcDNA CTCF = 1.63 ± 0.09; pcDNA-PrPC CTCF = 6.44 ± 0.12; pcDNA-PrPC + Glia CTCF = 2.82 ± 0.20; pcDNA-PrPC + PLC CTCF = 2.06 ± 0.15. pcDNA vs pcDNA-PrPC t = 7.40, mean diff. = −4.8, 95% CI of diff. = −6.88 to −2.72. pcDNA-PrPC vs pcDNA-PrPC+Glia t = 5.31, mean diff. = 3.6, 95% CI of diff. = 1.43 to 5.78. pcDNA-PrPC vs pcDNA-PrPC + PLC t = 6.02, mean diff. = 4.37, 95% CI of...
Enhanced cell death in the CA3 hippocampal region ΔF35 and ΔC4 mice compared to B6129 mice. Prnp\(^{0/0}\) ΔF35 (ΔF35) and Prnp\(^{0/0}\) ΔC4 (ΔC4) mice (Fig. 4a) were generated some years ago by nuclear injections of constructs into fertilized oocytes from B6129 Prnp\(^{Zrc1/Zrc1}\) mice. The number of copies of the construct was estimated as 25 for ΔC4 and 70 for ΔF35 mice. However, brain extracts obtained from these mice revealed similar levels of PrP−ΔC4 (Prnp\(^{+/+}\); PrP−ΔC4 = 0.81 ± 0.03; P = 0.4; CI = 95%; Mann-Whitney U test) to wild-type but lesser amounts of PrP−ΔF35 than wild-type (Prnp\(^{+/+}\); PrP−ΔF35 = 0.70 ± 0.021; P = 0.0048, CI = 95%; Mann-Whitney U test) (Fig. 4b and Supplementary Fig. 3). Nevertheless only ΔF35 mice showed cerebellar degeneration at around 60 days of life (Supplementary Fig. 4). Irrespective of the cell type, the expression of the truncated form may induce per se cell death in vivo as well as in vitro. Thus in the next experiments we treated these mice with 8 mg/kg b.w. of KA following the above-mentioned protocol. Behavioural results reported a similar evolution between ΔC4 (n = 11) and ΔF35 (n = 15)
Figure 4. KA-induced seizures and neurotoxicity in ΔC4 and ΔF35 mice. (a) Scheme of PrP<sup>C</sup> and N-terminal truncated forms overexpressed in B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>+/+</sup> ΔC4 and B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> ΔF35 transgenic mice. (b) Example of western blot detection of PrP<sup>C</sup> (6H4) expression in brain extracts obtained from untreated B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>+/+</sup> and B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup>, B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> ΔC4 and B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> ΔF35 mice. Tubulin was used as a loading control. (c) KA-induced seizure sensitivity in adult B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> ΔC4 mice in comparison to B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> and B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> ΔF35 mice (upper panel). KA-induced seizure sensitivity in 5–7 week-old B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> ΔF35 mice in comparison to B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> and B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> ΔF35 mice (lower panel). Bars represent the percentage of mice reaching each stage. (d) Fluoro-Jade B staining in hippocampal sections of B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>+/+</sup>, B6129 PrnP<sup>Zrcl/Zrcl</sup> PrnP<sup>−/−</sup> ΔF35 mice 24 hours after KA treatment (8 mg/kg b.w.). The quantification of the Fluoro-Jade B-positive cells in the CA3 region is shown in the right plot. Scale bars represent 200 μm. Abbreviations: DG, Dentate gyrus; CA1-3, hippocampal regions 1 and 3; h, hilus; gl, granule cell layer; ml, molecular layer; sr, stratum radiatum; slm, stratum lacunosum-moleculare; sl, stratum lucidum; sp, stratum pyramidale; so, stratum oriens. Asterisks indicate statistical significance (**P < 0.01, Bonferroni post hoc test).
mice compared to B6129 Prnp<sup>Zrchl/Zrchl</sup> (<i>n</i> = 35) and B6129 Prnp<sup>+/+</sup> (<i>n</i> = 30) (Fig. 4c and Supplementary Movie 4). However, the number of dead was greater in ΔC4 (<i>≈</i>38%) and ΔF35 (<i>≈</i>15%) compared to B6129 Prnp<sup>Zrchl/Zrchl</sup> Prnp<sup>0/0</sup> mice. These results also correlate with an increased presence of Fluoro-Jade B cells observed in histological sections of the hippocampus, especially in the CA3 region (<i>ΔF35</i> = 87.12 ± 0.41; <i>ΔC4</i> = 104.62 ± 0.088; B6129 Prnp<sup>Zrchl/Zrchl</sup> vs <i>ΔF35</i> <i>t</i> = 7.577; mean diff. = −73.75, 99% CI of diff. = −115.4 to −32.1. B6129 Prnp<sup>Zrchl/Zrchl</sup> vs <i>ΔC4</i> <i>t</i> = 9.375; mean diff. = −91.25, 99% CI of diff. = −132.9 to −49.6, Bonferroni post hoc test). (Fig. 4d).

Taken together, these results indicate that the expression of the truncated form including the octarepeat region (OR) with or without the Central Domain (CD) of PrP<sub>C</sub> potentiates the effects of the KA (<i>≤</i>10 mg/kg b.w.), and they highlight the octarepeat domain as a key candidate in the neuroprotective functions of PrP<sub>C</sub>.

Acute transfection of pcDNA-PrP<sub>ΔCD</sub> but not pcDNA-PrP<sub>ΔF35</sub> protects N2a cells from KA excitotoxicity in vitro.

In order to corroborate in vitro the participation of the OR in PrP<sub>C</sub>-mediated neuroprotection to KA, we performed a viability assay using N2a cells. Cells were transfected with vectors encoding either the full length of Prnp or two truncated forms lacking CD (PrP<sub>ΔCD</sub>, residues 95–133), which bridge the flexible amino proximal tail and the globular carboxy proximal domain, or else a longer deletion including the central domain (CD) plus the OR (PrP<sub>ΔF35</sub>, residues 32–134) (Fig. 5a). After transfection, levels of PrP<sub>C</sub> and its truncated forms in transfected cells were checked with western blotting (Fig. 5b and Supplementary Fig. 5). In addition, the expression of the truncated forms was strictly modulated to avoid inducing cell death during the experiments. Transfected cells were treated overnight with 5 mM KA and further processed to WST-1 assays. Colorimetric WST-1 assay showed that PrP<sub>C</sub> and PrP<sub>ΔCD</sub> transfection increased cell culture viability after KA treatment, an effect that was not observed in PrP<sub>ΔF35</sub> transfected cells (pcDNA = 0.54 ± 0.005; pcDNA-PrP<sub>C</sub> = 0.94 ± 0.06; pcDNA-PrP<sub>ΔCD</sub> = 1.047 ± 0.012 and pcDNA-PrP<sub>ΔF35</sub> = 0.88 ± 0.03. pcDNA vs pcDNA-PrP<sub>C</sub> <i>t</i> = 36.18; mean diff. = −0.4, 99% CI of diff. = −0.45 to −0.34, pcDNA vs pcDNA-PrP<sub>ΔCD</sub> <i>t</i> = 45.83; mean diff. = −0.506, 99% CI of diff. = −0.55 to 0.45. pcDNA-PrP<sub>ΔCD</sub> vs pcDNA-PrP<sub>ΔF35</sub> <i>t</i> = 15.08, mean diff. = 0.166, 95% CI of diff. = −0.1282 to 0.20).

Figure 5. KA-excitotoxicity in N2a cells transiently transfected with PrP<sub>C</sub> or PrP-ΔCD and PrP-ΔF35 constructs. (a) Scheme illustrating domain organization in PrP<sub>C</sub> and its related PrP<sub>ΔCD</sub> and PrP<sub>ΔF35</sub> mutants. (b) Example of western blot determination of the different PrP<sub>C</sub> constructs employed in the experiment. (c) WST-1 viability assay performed over N2a cells previously transfected with pcDNA3.1 (empty vector, pcDNA), pcDNA-PrP<sub>C</sub>, pcDNA-PrP<sub>ΔCD</sub> and pcDNA-PrP<sub>ΔF35</sub> and treated overnight with 5 mM KA. Data were normalized with untreated controls. A clear reduction in cell viability was observed in pcDNA-PrP<sub>ΔF35</sub> transfected cells when compared to those overexpressing pcDNA-PrP<sub>C</sub>. In contrast, pcDNA-PrP<sub>ΔCD</sub> transfected cells showed similar viability to pcDNA-PrP<sub>C</sub> transfected cells. Histograms represent the mean ± S.E.M. of three different experiments. Asterisks indicate statistical significance (**<i>P</i> < 0.05, ***<i>P</i> < 0.01, Bonferroni post hoc test).
including PrPC downregulation and detachment of the plasma membrane in neuronal primary cultures. Results have been published by other groups, who described an elevated threshold for epileptiform activity in Prnp\(^{0/0}\) hippocampal slices exposed to bicuculline, PTZ or zero-magnesium conditions. Similar discrepancies have been found when analysing neurotransmission-associated parameters in mice devoid of PrP\(^{C}\) (see77,78 for review). Besides the highly conserved hydrophobic domain, the flexible and unstructured C-terminal region of PrPC is also involved in the interaction of different regions of PrP\(^{C}\) with the plasma membrane through the GPI domain has been considered necessary to induce the clinical symptoms in GPI-negative transgenic mice (C57BL/10-PrP\(^{GPliss}\) mice)31,72. In fact, the injection of antibodies directed to the \(\alpha1\) and \(\alpha3\) domains of the PrP\(^{C}\) induces neurotoxic effects79. These results have also been corroborated in newly developed mice lacking the globular domain (FTgp1 mice)74. In fact, these results corroborate previous observations75. Taken together, these studies suggest that the proximity of the flexible tail (N-terminal domain) to the plasma membrane triggers intracellular oxidative stress responses leading to cell death74,77. Under this scenario we can consider that our data reinforce the idea that the integrity of the N-terminal domain is mandatory for neuroprotection (see below) as well as the notion that membrane interaction is a necessary part of the neuroprotective function reported in vivo.

The neuroprotective function of PrP\(^{C}\) depends on membrane anchoring. Our results indicate that the neuroprotective function of PrP\(^{C}\) against KA in N2a cells depends on membrane anchoring. The interaction of different regions of PrP\(^{C}\) with plasma membrane through the GPI domain has been considered necessary to induce the clinical symptoms in GPI-negative transgenic mice (C57BL/10-PrP\(^{GPliss}\) mice)31,72. In fact, the injection of antibodies directed to the \(\alpha1\) and \(\alpha3\) regions of the PrP\(^{C}\) induces neurotoxic effects79. These results have also been corroborated in newly developed mice lacking the globular domain (FTgp1 mice)74. In fact, these results corroborate previous observations75. Taken together, these studies suggest that the proximity of the flexible tail (N-terminal domain) to the plasma membrane triggers intracellular oxidative stress responses leading to cell death74,77. Under this scenario we can consider that our data reinforce the idea that the integrity of the N-terminal domain is mandatory for neuroprotection (see below) as well as the notion that membrane interaction is a necessary part of the neuroprotective function reported in vivo.

The neuroprotective function of PrP\(^{C}\) is abolished in the absence of the OR of PrP\(^{C}\). Structural analysis of PrP\(^{C}\) architecture has determined different functionally relevant domains in this protein (see77,78 for review). Besides the highly conserved hydrophobic domain, the flexible and unstructured
N-terminus region includes a copper binding site consisting of four tandem repeats of the sequence PHGGGGWQ, which seems to be involved in the endocytic process of the protein and copper homeostasis. In fact, a recent study indicates a relevant role of copper binding in the neuroprotective function of PrP modulating NMDA receptor. In our study we developed in vivo as well as in vitro tests to check the involvement of the OR domain as a mediator of PrP neuroprotective function in a model of KA treatment. The behavioural and histological data presented here by ΔC4 and ΔF35 mice revealed increased cell death in the hippocampus of the KA injected mice compared to B6129 Prnp0/0 (genetic background of these mice). In parallel, the CD is not involved in the neuroprotective functions of PrP at least in N2a cells) since their absence does not modify these properties when compared to full length PrP, in contrast to the absence of the OR regions.

The overexpression of PrP−ΔF35 in a B6129 PrnpZrchl/Zrchl Prnp0/0 background leads to cerebellar neurodegeneration, but not hippocampal degeneration (Supplementary Fig. 4). In contrast, ΔC4 mice (with similar background) do not show cerebellar or hippocampal degeneration, but when subjected to controlled ischemia show significantly greater oxidative stress damage when compared to wild type mice. This also happens when PrP−ΔF35 is overexpressed in HEK293 cells, leading to increased Caspase 3 activity in transfected cells and cell death. Truncated forms of PrP lacking the OR interfere with PrP endocytosis via clathrin-coated vesicles and beta-cleavage of PrP; respectively, thereby impairing the antioxidative functions of PrP. Thus it is reasonable to consider that cells with an intrinsic deficit in oxidative stress homeostasis may also be more prone to KA treatment if the appropriate KA receptors are also expressed, as happens in the hippocampus.

As indicated above, using antibody-mediated degeneration, Sonati et al., demonstrated that ligands directed to the α1 and α3 helices of the PrP globular domain induce cerebellar cell death by activating oxidative stress that can be overcome by deletions in the OR region. This also happens in F1tgpi mice lacking the α1-α3 helix region of the PrP. In addition, mice lacking the CD also reported white matter pathology and peripheral neuropathy. Surprisingly, the reported degeneration of the α1 helix could be reversed by coexpression of PrP lacking all octarepeats. These data are in contrast to a recent study indicating that the antibody ICSM18 (recognizing aa143–153 of PrP) does not induce cell death. In our experiments, we observed that cells transfected with PrP−ΔCD are able to overcome PrP-mediated cell death as are those transfected with full length PrP, in contrast to PrP−ΔF35 transfected N2a cells. Our in vitro experiments are different from the in vivo situation, since an effect of ligands in the CD regions is unlikely; rather, they suggest a parallel effect of KA excytotoxicity plus the homeostatic imbalance induced by the absence of the 32–93 region of the overexpressed PrP. Despite the existing data, the precise mechanism underlying OR-dependent neuroprotection remains to be elucidated in the above-mentioned studies.

In conclusion, our study dissects the effects of the intraperitoneal injection of various doses of KA in several Prnp mouse models and indicates that: i) PrP plays a role in neuroprotection in KA-treated cells and mice; ii) for this role PrP should be linked to the membrane; iii) polymorphisms of some unidentified “Prnp-flanking genes” play a parallel role to PrP in the KA-mediated responses in B6129 and B6.129 PrnpZrchl/Zrchl Prnp0/0 mice; and iv) the absence of the aa32–93 region negatively affects the neuroprotective function of PrP in KA-treated cells.

Methods
Reagents and antibodies
KA, glimepiride and phospholipase C were purchased from Sigma (Poole Dorset, UK). Fluoro-Jade B was from Millipore (Billerna, MA), SYBR green (Applied Biosystems, USA) and WST-1 reagents were from Roche (Basel, Switzerland). Lipofectamine plus was from Invitrogen (Carlsbad, CA). The following antibodies were used in this study: Anti-PrP SAF61 mouse monoclonal antibody (1:1000 diluted) antibody was from Spi-Bio (Cayman Chemical, Massy, France) and anti-PrP 6H4 mouse monoclonal antibody (1:5000 diluted western blotting and 1:250 immunocytochemistry) antibody was from Prionics (Schlieren, Switzerland). Mouse monoclonal antibody anti-tubulin (1:10000 diluted) was from Sigma (Poole Dorset, UK) and rabbit-raised polyclonal antibody against GFAP (1:500 diluted) was from Millipore (Billerna, MA).

Animals
Adult male C57Bl/6J29-kvPrnp0/0 (B6129 PrnpZrchl/Zrchl Zurich I) mice were purchased from the European Mouse Mutant Archive (EMMA, Monterotondo, Italy). The FVB/N animals were either wild-type or homozygous for the deletion of Prnp gene. 129/Ola PrnpΔf35/Δf35 and 129/Ola Prnp0/0 were obtained from Dr. J. Manson (Edinburgh). B6129 PrnpZrchl/Zrchl mice were backcrossed for 8 generations to obtain 6–7% of 129 microsatellites (B6.129 PrnpZrchl/Zrchl). Transgenic B6129 Prnp0/0 PrPΔC4 89 and B6129 Prnp0/0 PrPΔF35 53 were obtained from Prof. A. Aguzzi. To avoid putative background-specific differences between mice, all of the experiments were conducted using littermates derived from heterozygous (Prnp0/0 ΔF35+) and Prnp0/0 parents. Specific primers for Prnp genotyping were designed in our laboratory based on the original P10 and P3 primers described elsewhere. PrPΔf35 transgene was detected by using the primers 5′-CTTTGGACTCTTCTTGATCCGGGTGACGC-3′ and 5′-CAACCGAGCTGAAGCATTCTGCCT-3′ set of primers and PrPΔC4 with 5′-GGCTGGGCTTGGTTCACTATTAGG-3′ and 5′-CAACCGAGCTGAAGCATTCTGCTC-3′. Tg44/+ mice expressing anchorless PrP51 on a C57BL/10 background were obtained from Dr. Bruce Chesebro (Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT 59840,
USA). A total of 83 litters (193 animals) were used in the present study. All experiments were performed under the guidelines and protocols of the Ethical Committee for Animal Experimentation (CEEA) of the University of Barcelona, and the protocol for the use of animals in this study was reviewed and approved by the CEEA of the University of Barcelona (CEEA approval #115/11 and 141/15).

**KA administration in mice and seizure analysis.** Convulsive non-lethal seizures in mice were induced by administration of KA in a multiple dose protocol. Fresh KA solution was prepared for each experiment. Animals were weighed and intraperitoneally injected with 8 or 10 mg/kg KA (b.w.) dissolved in 0.1 M PBS, pH 7.2. At 0 min, 30 min and 60 min. In parallel mice, 0.1 M PBS, pH 7.2 was injected as control (vehicle). Adult (2–3 months old) animals were used in all experiments except for B6129 Prnp<sup>−/−</sup> Zrchl/Zrchl, B6129 Prnp<sup>−/−</sup> Prp<sup>ΔC</sup>, B6129 Prnp<sup>−/−</sup> Prp<sup>ΔF35</sup>, FVB/N Prnp<sup>−/−</sup> mouse strains and its respective controls) and in the Centro de Investigación en Sanidad Animal (CISA-INIA) by P.C, P.A-C., A-C, P.C. post hoc test (Multiple comparison test) or Mann-Whitney U test (non-parametric test using Prism 5.0c (Mac OsX, Grahpad). Data are presented as mean ± standard error of the mean (S.E.M.). A value of **P < 0.01 was considered statistically significant.

**Western blotting.** Brain samples from non-treated mice were homogenized (10% wt/vol) in ice-cold lysis buffer—50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% (wt/vol) Triton X-100, 0.5% (wt/vol) Nonidet P-40 (IGEPA; Sigma), glycerol 10%, 1 mM EDTA, 1 mM EGTA, and protease and phosphatase inhibitors—using a motor-driven, glass-Teflon homogenizer in ice, and then centrifuged at 15,000g for 20 min. Protein concentration was quantified with BCA kit (Pierce). Protein extracts were boiled in Laemmli sample buffer at 100 °C for 5 min. Equal amounts of total protein were separated with 6–10% SDS-PAGE electrophoresis, electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore), and probed with indicated antibodies. Visualization of bound antibodies was performed using goat anti-mouse HRP (1:4000 diluted; Dako, Glostrup, Denmark) and the ECL Plus kit (Amersham-Pharmacia Biotech, GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

**Densitometry and statistical processing of processed films.** For quantification, developed films were scanned at 2400 × 2400 dpi (8000 MICROTEK high quality film scanner), and the densitometric analysis of the different PrP<sup>C</sup> bands was performed in each case using Quantity One Image Software Analysis (BioRad). Each densitometric value of PrP<sup>C</sup> and truncated form ΔC, ΔF35 and ΔCD (0–255 gray scales) was normalized with the corresponding Tubulin densitometric values (0–255 gray scale). Three different experiments were used in each analysis unless specified. The statistical analysis of the obtained data was performed using Bonferroni post hoc test (Multiple comparison test) or Mann-Whitney U non-parametric test using Prism 5.0c (Mac OsX, Grahpad). Data are presented as mean ± standard error of the mean (S.E.M.). A value of **P < 0.01 was considered statistically significant.

**Fluoro-Jade B staining.** Mice were perfused with 4% paraformaldehyde dissolved in 0.1 phosphate buffer, pH 7.3 24 hours after the first KA injection, post-fixed overnight in the same fixative, and cryoprotected in 30% sucrose. 30 μm-thick coronal brain sections were obtained in a freezing microtome (Leica, Wetzlar, Germany). Sections containing dorsal hippocampus (Bregma = −1.2 to −1.9) were rinsed for 2 h in 0.1 M Tris, pH 7.4, mounted and air dried at room temperature overnight. The next day, sections were pre-treated for 3 min in absolute ethanol, followed by 1 min in 70% ethanol and 1 min in distilled water. They were then oxidized in a solution of 0.06% KMnO4 for 15 min. After three rinses of 1 min each in distilled water, the sections were incubated for 30 min in 0.001% Fluoro-Jade B (Chemicon) containing 0.01% of DAPI (Sigma) in 0.1% acetic acid. The slides were rinsed in deionized water for 3 min each, dried overnight, cleared in xylene, cover-slipped with Eukitt (Merck, Darmstadt, Germany) and examined using an Olympus (Hamburg, Germany) BX61 epifluorescence microscope. The statistical analysis of the obtained data was performed using Bonferroni post hoc test (Multiple...
comparison test) using Prism 5.0c (Mac OsX, Grahpad). Data are presented as mean ± standard error of the mean (S.E.M.). A value of ***$P < 0.01$ was considered statistically significant.

**Histology and immunofluorescence.** For histology, mice were perfused with phosphate buffered 4% paraformaldehyde, pH 7.3 24 hours after the first KA injection, post-fixed overnight in the same fixative, and cryoprotected in 30% sucrose as above. A freezing microtome (Leica, Wetzlar, Germany) was used to obtain 30 μm-thick coronal sections, which were rinsed in 0.1 M PBS before 1 hour’s incubation at room temperature in 0.1 M PBS containing 0.2% gelatin, 10% normal goat serum, 0.2% glycine, and 0.2% Triton X-100. Sections were then incubated overnight at 4°C with indicated primary antibodies. After washing in 0.1 M PBS containing 0.2% Triton X-100, sections were incubated with goat anti-rabbit Alexa Fluor 568-tagged secondary antibody (1:200 diluted; Molecular Probes, Eugene, OR, USA), washed in 0.1 M, PBS and mounted in Fluoromount (Vector Labs, Burlingame, CA, USA). Immunohistochemical controls, which included omission or substitution of primary anti-GFAP antibody by either normal rabbit serum prevented immunostaining. For quantification of GFAP-positive astrocytes in the stratum radiatum of the dorsal hippocampal region, immunoreacted sections (5 sections of each mouse, $n = 5$ mice per genotype) were photodocumented with an Olympus BX61 fluorescence microscope equipped with a cooled DP12L camera. Photomicrographs were obtained using a 40X objective with identical time exposure (100–150 ms) between preparations from each wild-type and respective knockout mouse. No modifications were applied to the obtained pictures. Numbers of GFAP-expressing cells were determined by counting positive cells in five frames (250 × 200 μm) corresponding to the hippocampal CA1-3 regions of five mice of each genotype. Data were expressed as mean ± standard error of the mean (S.E.M). The statistical analysis of the obtained data was performed using Mann–Whitney U non-parametric test using Prism 5.0c (Mac OsX, Grahpad). A value of $P < 0.01$ was considered statistically significant.

**RT-qPCR.** Total RNA from hippocampal samples obtained from treated (6 h after KA-administration) and non-treated mice was purified with the mirVana isolation kit (Ambion, Austin, TX, USA) and used to make the single-stranded cDNAs required as templates for the RT-qPCR amplification. Sets of primers used in this study were: for TNFα 5′- AGCAAAAACCAAGTGGGAGA- 3′ and 5′-GCTGCCACCTAGTTGGTTG- 3′; and for ILβ 5′- TTGTGGCTGGAGGACGTGT- 3′ and 5′- AACGTCACACCCAGCAGT- 3′. The reaction was performed with the Roche LightCycler 480 detector, using 2x SYBR Green Master Mix (Roche) as reagent, as indicated by the manufacturer. Amplification protocol consisted of a denaturation-activation cycle (95°C for 10 min) followed by 40 cycles of denaturation-annealing-extension (95°C, 15 sec; 60°C, 40 sec; 72°C, 5 sec; 98°C, continuous). LightCycler 480 software was used for mRNA quantification. The data were analysed using the ΔΔGct method, which provides the target gene expression values as fold changes in the problem sample compared with a calibrator sample. Both problem and calibrator samples were normalized by the relative expression of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). These analyses developed 3 different samples. The statistical analysis of the obtained data was performed using Mann–Whitney U non-parametric test using Prism 5.0c (Mac OsX, Grahpad). Data are presented as mean ± standard error of the mean (S.E.M.). A value of ***$P < 0.01$ was considered statistically significant.

**Cell culture and treatments.** The murine neuroblastoma cell line Neuro2a (N2a) expressing low levels of PrPc was grown at 37°C, 5.5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 g/L glucose, 10% fetal bovine serum (FBS) and antibiotics (Invitrogen-Life Technologies, Barcelona, Spain). For vector transfection, cells were plated at 1 × 105 cells/well in a 24-well plate and transiently transfected the next day using Lipofectamine 2000 reagent in Optimem medium, as indicated by the manufacturer (Invitrogen-Life Technologies). Four hours after transfection, cells were washed and the medium was replaced with DMEM containing 10% FBS. PrPc expression after transfection was checked with western blot analysis. In a first set of experiments, N2a cell cultures were transiently transfected with pcDNA and pcDNA-PrPc and maintained for 48 hours. Total RNA from hippocampal samples obtained from treated (6 h after KA-administration) and non-treated mice was purified with the mirVana isolation kit (Ambion, Austin, TX, USA) and used to make the single-stranded cDNAs required as templates for the RT-qPCR amplification. One hour later, 5 mM KA dissolved in 0.1 M PBS was added to the media. After treatment, cultures were rinsed twice in KA-free culture medium, and cell viability was determined with WST-1 viability assay (see below). In parallel, non-permeabilized cells were processed to PrPc detection by immunofluorescence using the 6H4 antibody and a goat anti-mouse Alexa Fluor 488–tagged secondary antibody (1:200 diluted; Molecular Probes). For fluorescence quantification of cell-bound Alexa Fluor 488, immunoreacted cultures (n = 10 per experimental group) were photodocumented with an Olympus BX61 + DP12L camera. Photomicrographs were obtained using a 20X objective with identical time exposure (250–300 ms) for preparations from each group. No modifications were applied to the obtained pictures. Fluorescence intensity was determined using ImageJ by measuring the corrected total cell fluorescence (CTCF) as: CTCF = integrated density – (area of selected N2a measured cells × mean fluorescence of background). Data were expressed as mean ± standard error of the mean (S.E.M). The statistical analysis of the obtained data in these experiments was performed using Bonferroni post hoc
test (Multiple comparison test) using Prism 5.0c (Mac OsX, Grahpad). A value of $P < 0.05$ was considered statistically significant. These experiments were repeated four times. In a second set of experiments, N2a cells were transfected with pcDNA3.1, pcDNA3.1-PrP<sup>C</sup>, pcDNA3.1-PrP<sup>∆CD</sup> and pcDNA3.1-PrP<sup>∆F35</sup>. KA (5 mM) treatment was carried out on serum-deprived cells 24 h after transfection. Cell viability was determined using a commercially available WST-1-based assay. Cell cultures were incubated with WST-1 reagent for 2 hours. Then absorbance at 450 nm was measured in a multiwell plate reader (Merck ELISA System MIO5). Data were normalized with A450 in untreated controls. pcDNA3.1-PrP<sup>C</sup>, pcDNA3.1-PrP<sup>∆CD</sup> and pcDNA3.1-PrP<sup>∆F35</sup> were kind gifts from Prof. D. Harris (Boston University) and Prof. A. Aguzzi (University Hospital of Zurich). These experiments were repeated five times. The statistical analysis of the obtained data in these experiments was performed using Bonferroni post hoc test (Multiple comparison test) using Prism 5.0c (Mac OsX, Grahpad). Data are presented as mean ± standard error of the mean (S.E.M.). Values of **$P < 0.05$ and ***$P < 0.01$ were considered statistically significant.

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Author Contributions

P.C., F.LL, A.M-A., P.A.-C., R.G. and J.C.E. performed most of the experiments. I.F., J.M.T., G.L. and J.A.D.R. designed the project and wrote the manuscript.

Additional Information

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