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The interaction between prion protein and laminin modulates memory consolidation

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Abstract

Cellular prion protein (PrP^c) has a pivotal role in prion diseases. PrP^c is a specific receptor for laminin (LN) γ1 peptide and several lines of evidence indicate that it is also involved in neural plasticity. Here we investigated whether the interaction between PrP^c and LN plays a role in rat memory formation. We found that post-training intrahippocampal infusion of PrP^c-derived peptides that contain the LN binding site ($PrP^c_{163-182}$ and $PrP^c_{173-192}$) or of anti-PrP^c or anti-LN antibodies that inhibit PrP^c–LN interaction impaired inhibitory avoidance memory retention. The amnesic effect of anti-PrP^c antibodies and $PrP^c_{173-192}$ peptide was reversed by co-infusion of a LN γ1 chain-derived peptide containing the PrP^c-binding site, suggesting that PrP^c–LN interaction is indeed crucial for memory consolidation. In addition, $PrP^c_{173-192}$ peptide and anti-PrP^c or anti-LN antibodies also inhibited the activation of hippocampal cAMP-dependent protein kinase A (PKA) and extracellular regulated kinase (ERK1/2), two kinases that mediate the up-regulation of signaling pathways needed for consolidation of inhibitory avoidance memory. Our findings show that, through its interaction with LN, hippocampal PrP^c plays a critical role in memory processing and suggest that this role is mediated by activation of both PKA and ERK1/2 signaling pathways.

Introduction

Cellular prion protein (PrP^c) is a cell surface, glycosylphosphatidylinositol-anchored protein that is abundantly expressed in neurons. Evidence indicates that conversion of PrP^c into its abnormal conformer, the scrapie prion protein (PrP^{sc}), causes transmissible spongiform encephalopathies (Prusiner *et al.,* 1998). PrP^c has been associated with regulation of ion transport, synaptic transmission and neuritogenesis, suggesting a role in neuronal plasticity (Reviewed by Martins & Brentani, 2002). In fact, the participation of PrP^c in learning and memory processing has been postulated (Nishida *et al*., 1997; Martins & Brentani, 2002; Wickelgren, 2004; Shorter & Lindquist, 2005) but, except for a few reports (Coitinho *et al*., 2003; Criado *et al.,* 2005), conclusive evidence is still lacking. Interestingly, a PrP^c polymorphism at codon 129 has been associated with early cognitive decline in humans (Croes *et al*., 2003). Additionally, adults presenting methionine in homozygosis or heterozygosis at codon 129 exhibit better long-term memory (LTM) than those with valine in this codon (Papassotiropoulos *et al*., 2005).

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Laminins (LNs) are components of the extracellular matrix formed by heterotrimeric molecules composed by α, β and γ chains (Colognato & Yurchenco, 2000). LN-10 (α5β1γ1) is abundantly expressed in the hippocampus (Indyk *et al*., 2003), where the γ1 chain has a critical role in axonal regeneration (Grimpe *et al*., 2002). We have shown that PrP^c is a receptor for a decapeptide (RNIAEIIKDI) at the C-terminus of the LN γ1 chain (Graner *et al*., 2000a). Thus, neuritogenesis by hippocampal cultures in the presence of this peptide was inhibited by anti-PrP^c antibodies. Furthermore, no neuritogenesis was elicited by LN-γ1 chain peptide in neurons obtained from PrP^c gene (*Prnp*) null mice, indicating that a PrP^c–LN association is involved in neural plasticity (Graner *et al*., 2000a).

Memories are stabilized through a post-encoding consolidation process that makes them resistant to change and interference from competing or disrupting factors in the absence of further rehearsal (McGaugh & Izquierdo, 2000). Step-down inhibitory avoidance (IA) is a well-known animal model for aversive learning in which stepping-down from a platform placed in a particular context is paired with a footshock (Bevilaqua *et al*., 1999; Bonini *et al*., 2003; Cammarota *et al*., 2005a). After one training session, animals learn to refrain from stepping-down to the grid when placed again on the training box platform. Consolidation of IA memory requires functional integrity of the hippocampal formation and activation of different neurotransmitter

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receptors for synaptic remodeling and morphological changes necessary for lasting storage of the mnemonic trace (Izquierdo *et al*., 2004). It is not clear which are the mechanisms coupling up-regulation of neurotransmitter receptors to gene expression during learning, although evidence suggests that cAMP-dependent protein kinase (PKA) and extracellular regulated kinase (ERK1/2)-dependent up-regulation of the cAMP response element binding protein (CREB) family transcription factors plays a role in those events (Bernabeu *et al*., 1997; Cammarota *et al*., 2000).

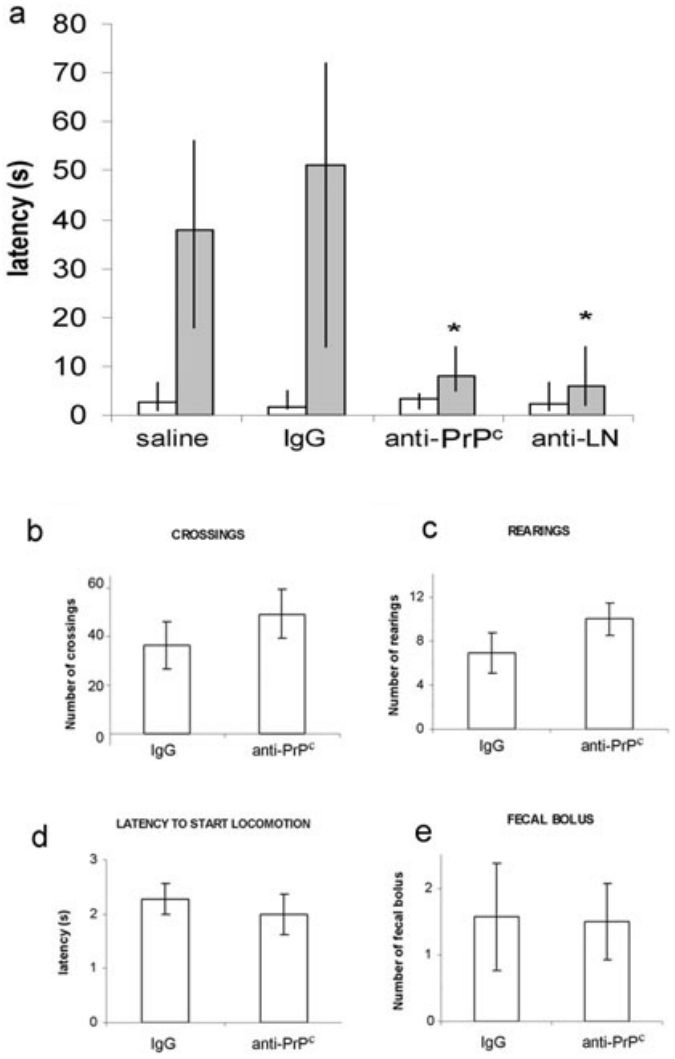

FIG. 1. Anti-PrP^c and anti-LN antibodies impair memory consolidation whereas no effect was observed in exploratory or locomotor activities. (a) Surgically implanted male Wistar rats were submitted to the previously described (Bernabeu *et al*., 1997; Izquierdo *et al*., 1998) step-down inhibitory avoidance task (IA). Latency to step down in the training trial was measured (white bars), and immediately after training animals received a bilateral infusion of anti-PrP^c IgG (1.8 μg/μL), anti-LN IgG (0.18 μg/μL) or control rabbit IgG (1.8 μg/μL) into CA1 area of the hippocampus. Memory was evaluated during non-reinforced session carried out 24 h post-training (grey bars). Data are shown as median (interquartile ranges) of latencies to step-down. Statistical comparisons were performed using Kruskall–Wallis followed by Dunn's *post-hoc* test. *Post-training anti-PrP^c or anti-LN vs. IgG, d.f. = 3, $P < 0.01$. (b–e) Performance of rats during 5 min exploration of an open field. Values are means ± SEM number of crossings, rearings, latency to start locomotion and fecal bolus, respectively, $n = 11$ animals per group. Comparisons between groups were made by unpaired *t*-test and no significant differences were found.

Here, we demonstrate the relevance of PrP^c–LN interaction for memory consolidation by blocking the proteins *in locus* and measuring retention of memory for a one-trial inhibitory avoidance task in rats. In addition, we show that the effect of PrP^c–LN interaction on memory is mediated by PKA and ERK1/2 signaling pathways.

Materials and methods

Animals

Male Wistar rats (3 months of age, 250–280 g) from our own breeding stock were used. Animals were housed in plastic cages and maintained at a constant temperature of 22–23 °C under a 12-h light/dark cycle (lights on at 07:00 h) with water and food freely available. A group of rats were bilaterally implanted under deep thionembutal anesthesia with 27-gauge guides aimed 1.0 mm above the CA1 region of the dorsal hippocampus in accordance with coordinates taken from the atlas of Paxinos *et al*. (1985) (A −4.2, L ±3.0, V 1.4). After surgery, the animals were allowed to recover for 5 days before submitting them to any other procedure. All efforts were made to reduce the number of animals used. All experiments were conducted strictly in accordance with the *Principles of Laboratory Animal Care* (NIH publication 85-23, revised 1996). This study was approved by the Committee for Ethics in Use of Animals (CEUA) from Fundação Antonio Prudente/Hospital do Câncer.

Inhibitory avoidance training

Rats were trained in a one-trial, step-down IA paradigm, a hippocampal-dependent learning task in which stepping-down from a platform present in a given context is associated with a footshock resulting in an increase in step-down latency (Cammarota *et al*., 2004, 2005b,c). The IA training apparatus was a 50 × 25 × 25-cm Plexiglas box with a 5-cm-high, 8-cm-wide and 25-cm-long platform on the left end of a series of bronze bars that constitutes the floor of the box. During training, animals were gently placed on the platform facing the left rear corner of the training box. When rats stepped down and placed their four paws on the grid they received a 2-s, 0.5-mA footshock immediately followed by a bilateral infusion of saline, antibodies and/or peptides in a total volume of 0.5 μL per side. Infusions were carried out over 60 s, first on one side and then on the other; the infusion cannula was left in place for a further 60 s to minimize backflow.

TABLE 1. Effect of intrahippocampal infusion of IgG, anti PrP^c or anti-LN on plus-maze performance

	Group		
Behavior	IgG	Anti-PrP^c	Anti-LN
Open arms			
Number of entries	5.7 ± 0.8	6.4 ± 1.1	7.1 ± 0.8
Permanency (s)	1.2 ± 0.2	1.5 ± 0.4	1.3 ± 0.5
Closed arms			
Number of entries	7.6 ± 1.3	7.2 ± 1.4	6.8 ± 1.5
Permanency (s)	3.3 ± 0.3	3.2 ± 0.5	3.5 ± 0.3

Animals received IgG, anti-PrP^c or anti-LN antibody infusions 15 min before sessions. Data are means ± SEM of the number of entries or permanency in the open and closed arms during a 5-min observation period. Statistical analyses were performed by ANOVA followed by Tukey's HSD *post-hoc* test. Groups were not statistically different.

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Step-down IA memory retention was evaluated in a test session carried out 24 h after training. At test, trained animals were put back on the training box platform until they stepped down to the grid. The latency to step-down during the test session was taken as an indicator of memory retention.

Two to 4 h after the test session animals received 0.5 μL of a 4% methylene-blue solution through the implanted cannulae and the extension of the dye 30 min thereafter was taken as indicative of the presumable diffusion of the vehicle or drug previously given. Only data from animals with correct cannulae implants were included in the statistical analyses.

A ceiling of 180 s was imposed to step-down latencies during retention tests and this variable neither follows a normal distribution nor fulfills the assumption of homoscedasticity. Data are presented as median ± interquartile range and were analysed using the Kruskal–Wallis non-parametric test followed by Dunn's *post-hoc* comparisons.

Open field and plus maze

To analyse their exploratory and locomotor activities, rats were placed on the left rear quadrant of a 50 × 50 × 39-cm open field with black polywood walls and a brown floor divided into 12 equal squares. The

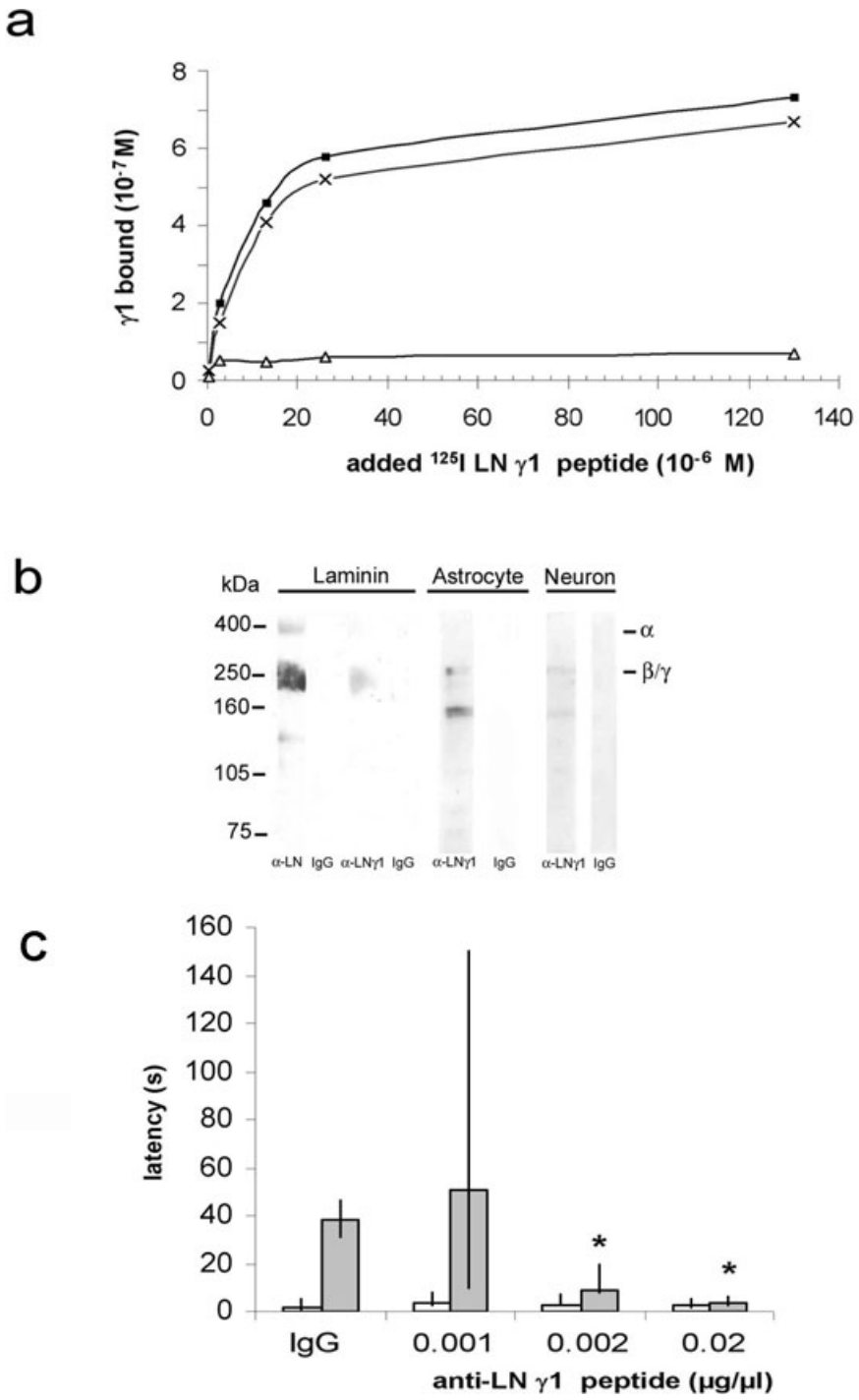

FIG. 2. LN domain that interacts with PrP^c is involved with memory consolidation. (a) LN γ1 chain peptide binds PrP^c in a specific high-affinity manner. ^{125}I-LN γ1 chain peptide was incubated with adsorbed His_6-PrP^c in the absence (total) or presence of unlabeled LN γ1 chain peptide (non-specific). Non-specific (open triangles) was subtracted from the total binding (closed squares) to yield His_6-PrP^c-specific binding to ^{125}I-LN γ1 chain peptide (crosses). (b) Characterization of the anti-LN γ1 peptide antibody. Purified laminin 1 and extracts from astrocyte and neuronal primary cultures were submitted to SDS-PAGE and immunoblotted with anti-LN or anti-LN γ1 chain peptide antibodies. Laminin chains α (450 kDa), β and γ (both around 240 kDa) are labeled on the right. (c) Antibodies against LN γ1 chain peptide inhibited memory retention. Experiments were performed as described in Fig. 1a. Latency to step down in the training trial was measured (white bars), animals received a bilateral infusion of IgG anti-LN γ1 peptide at the concentrations indicated or non-immune IgG (0.2 μg/μL) into CA1 area of the hippocampus. Memory consolidation was evaluated during a non-reinforced session carried 24 h post-training (grey bars). Statistical comparisons were performed using Kruskall–Wallis followed by Dunn's *post-hoc* test. *Post-training 0.02 or 0.002 μg/μL anti-LN γ1 peptide vs. post-training IgG, d.f. = 3, $P < 0.01$.

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number of line crossings and the number of rearings were measured over 5 min and taken as an indicative of locomotor and exploratory activities, respectively. To evaluate their anxiety state, an independent set of rats were exposed to an elevated plus maze exactly as previously described (Pellow *et al.*, 1985). The total number of entries into the four arms, the number of entries and the time spent in the open arms were recorded over a 5-min session.

Fifteen minutes before exposure to the open field or the plus maze, the animals received bilateral 0.5-μL infusions of purified IgG from anti-PrP^c, anti-LN or non-immune serum into the CA1 region of the dorsal hippocampus. Data are shown as mean ± SEM number of crossings, rearings, fecal bolus and latency to initiate locomotion. Open field groups were compared using unpaired *t*-tests while ANOVA followed by Tukey-HSD *post-hoc* test analyses were performed to compare plus maze groups.

Drugs

Anti-PrP^c IgG was raised in rabbit against the recombinant GST-PrP^c fusion protein and IgG against recombinant GST was used as the control IgG. This antibody has been tested in flow cytometry and immunofluorescence in non-permeabilized cells, demonstrating that it recognizes PrP^c in its native form at the cell surface (Graner *et al.*, 2000a). Anti-LN IgG was raised in rabbit against LAMININ 1; this antibody recognizes and blocks LN at the extracellular matrix (Line *et al.*, 1990; Giordano *et al.*, 1994). Monoclonal antibodies 8H4 and 8B4 (Cui *et al.*, 2003), which recognize PrP^c residues 175–185 and 34–45, respectively, were kindly provided by Dr Man-Sun Sy (Case Western Reserve University, USA). Rabbit IgG anti-LN γ1 chain derived peptide from amino acids 1575–1584 (RNIAEIIKDI) was produced by Bethyl Inc. (TX, USA). PrP^c peptides, laminin γ1 chain derived peptide (RNIAEIIKDI) and laminin γ1 scrambled peptide (IRANIEIKID) were from Neosystem (Strasbourg, France).

Expression and purification of recombinant PrP^c

The expression vector containing the cDNA fragment encoding amino acids 23–231 of the mouse PrP^c protein was kindly provided by Dr Ralph Zahn (Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, Switzerland). His$_6$-PrP^c expression and purification were performed as previously described (Zanata *et al.*, 2002).

PrP^c–LN γ1 peptide binding and competition assays

His$_6$-PrP^c (4 μg) was immobilized in polystyrene wells (Immulon 2) and non-specific sites blocked with 1% bovine serum albumin (BSA) for 2 h at room temperature. Increasing concentrations of ^{125}I-LN γ1 chain peptide (RNIAEIIKDI) linked to BSA (labeled as described by Chiarini *et al.*, 2002) with specific activity of 7×10^5 c.p.m./μg were added to the wells and incubated for 16 h at 4 °C. After extensive washing, incorporated radioactivity was measured (total binding curve). In parallel, His$_6$-PrP^c (4 μg) was incubated with ^{125}I-LN γ1 chain peptide-BSA plus five-fold excess unlabeled LN γ1 chain peptide-BSA (non-specific binding curve). Specific binding was obtained by subtraction of non-specific from total values.

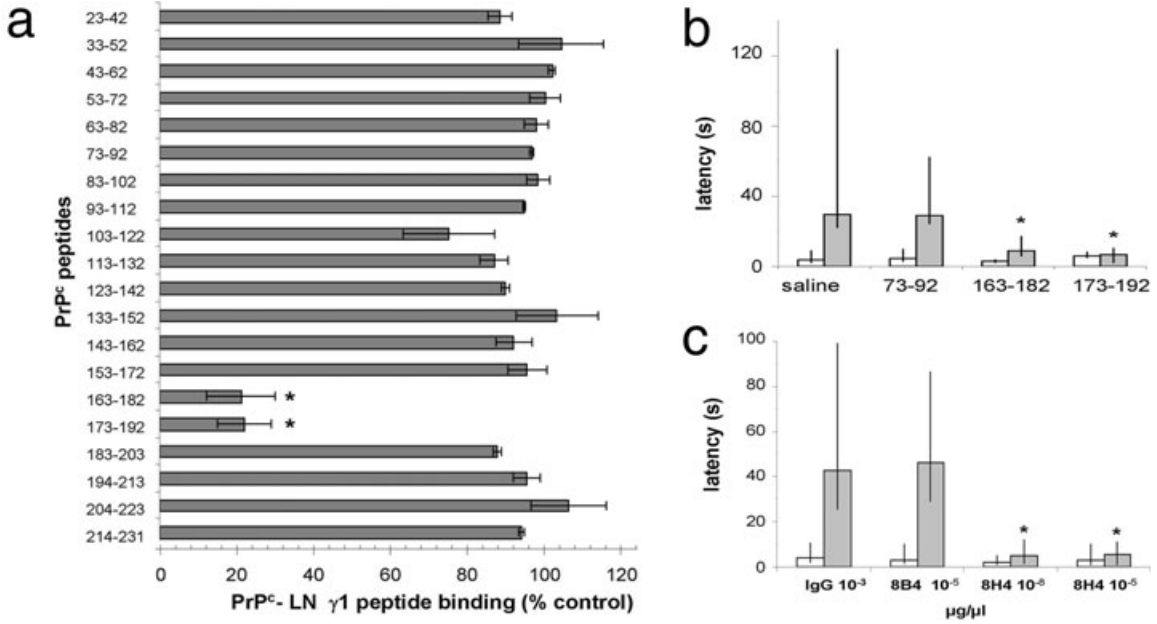

FIG. 3. PrP^c domain that interacts with LN is related to memory consolidation. (a) Mapping LN binding site domain using a competition assay with PrP^c peptides. Peptides (3.8×10^{-5} M) covering the mouse PrP^c (23–231) sequence were pre-incubated with 1.2×10^{-6} M ^{125}I-LN γ1 chain peptide followed by incubation in His$_6$-PrP^c adsorbed wells. After extensive washing, radioactivity was measured. Total His$_6$-PrP^c and ^{125}I-LN γ1 peptide binding was set as 100% and the results expressed as the relative percentage of binding produced by competition with each peptide. Results represent mean ± SEM of three independent experiments and statistical analyses were performed by ANOVA followed by Tukey's HSD *post-hoc* test. *Peptide 163–182 or peptide 173–192 vs. total binding (100%), d.f. = 20, $P < 0.0001$. (b) PrP^c peptides representing the LN binding site inhibited memory retention. Experiments were performed as in Fig. 1a. Latency to step down in the training trial was measured (white bars) and animals received intrahippocampal infusion of PrP^c peptides 73–92, 163–182 or 173–192 (0.2 μg/mL). Memory consolidation was evaluated during a non-reinforced session carried 24 h post-training (grey bars). Statistical comparisons were performed using Kruskall–Wallis followed by Dunn's *post-hoc* test. *Post-training PrP^c 163–183 or PrP^c 173–172 vs. post-training saline, d.f. = 3, $P < 0.01$. (c) Monoclonal antibody against the PrP^c domain which interacts with LN blocked memory retention. Experiments were performed as in Fig. 1a. Latency to step down in the training trial was measured (white bars) and animals received infusion of purified IgG monoclonal antibodies 8H4, 8B4 or control IgG. Memory retention was evaluated during a non-reinforced session carried 24 h post-training (grey bars). Statistical comparisons were performed using Kruskall–Wallis followed by Dunn's *post-hoc* test. *Post-training 8H4 at 10^{-8} μg/μL or 10^{-5} μg/μL vs. post-training IgG, d.f. = 3, $P < 0.01$.

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Competition assays were performed using PrP^c synthetic peptides and antibodies. Synthetic mouse PrP^c peptides (Zanata *et al*., 2002), 3.8×10^{-5} M, anti-PrP^c IgG or non-immune serum IgG were pre-incubated with 1.2×10^{-6} M ^{125}I-LN γ1 chain peptide-BSA for 3 h at room temperature. Then, the reagents were added to the wells containing 4 μg adsorbed His_6-PrP^c and incubated for 16 h at 4 °C. After extensive washing, incorporated radioactivity was determined using a gamma counter. The PrP^c ^{125}I-LN γ1 chain peptide total binding was considered to be 100% and those obtained in the presence of peptides or antibodies (IgGs) were relative to it.

Data are expressed as mean ± SEM obtained from at least three independent experiments. In the competition assay with PrP^c peptides (see Fig. 3a) statistical analyses were performed by ANOVA followed by Tukey's HSD *post-hoc* test. In Fig. 4b the two groups treated with the same concentration of IgG or anti-PrP^c were compared using an independent sample Student's *t*-test.

Immunoblotting

Cultured neurons or glia were lysed (PBS plus 1% NP-40) and 200 μg of proteins was submitted to SDS-PAGE, along with 2 μg of EHS-purified LN. Proteins were transferred to a nitrocellulose membrane and immunoblotting was performed with rabbit anti-LN IgG (1 : 1000) or rabbit anti-LN γ1 chain derived peptide (1 : 3000) in TBS plus 0.05% Tween-20.

Sample preparation and kinase assays

Naïve rats or those that were infused with saline, antibodies and/or peptides were killed by decapitation 10 min or 2 h after training and used for the enzymatic assays. Hippocampi were dissected and nuclear extracts prepared as previously described (Cammarota *et al*., 2000). PKA activity of each sample was determined using an assay system (Invitrogen, CA, USA) according to the manufacturer's instructions. Nuclear extracts from each sample were also used to immunoprecipitate active ERK1/2 using an immobilized phospho-p44/42 MAP kinase monoclonal antibody (Cell Signaling non-radioactive kit, MA, USA). ERK1/2 activity was evaluated by incubation with Elk-1 substrate, followed by electrophoresis and immunoblotting with anti-phospho Elk-1 antibody (1 : 1000, Cell Signaling). Densitometric analyses were performed using a MCID Image Analysis System (5.02 v, Image Research). Enzyme activities of each treatment were

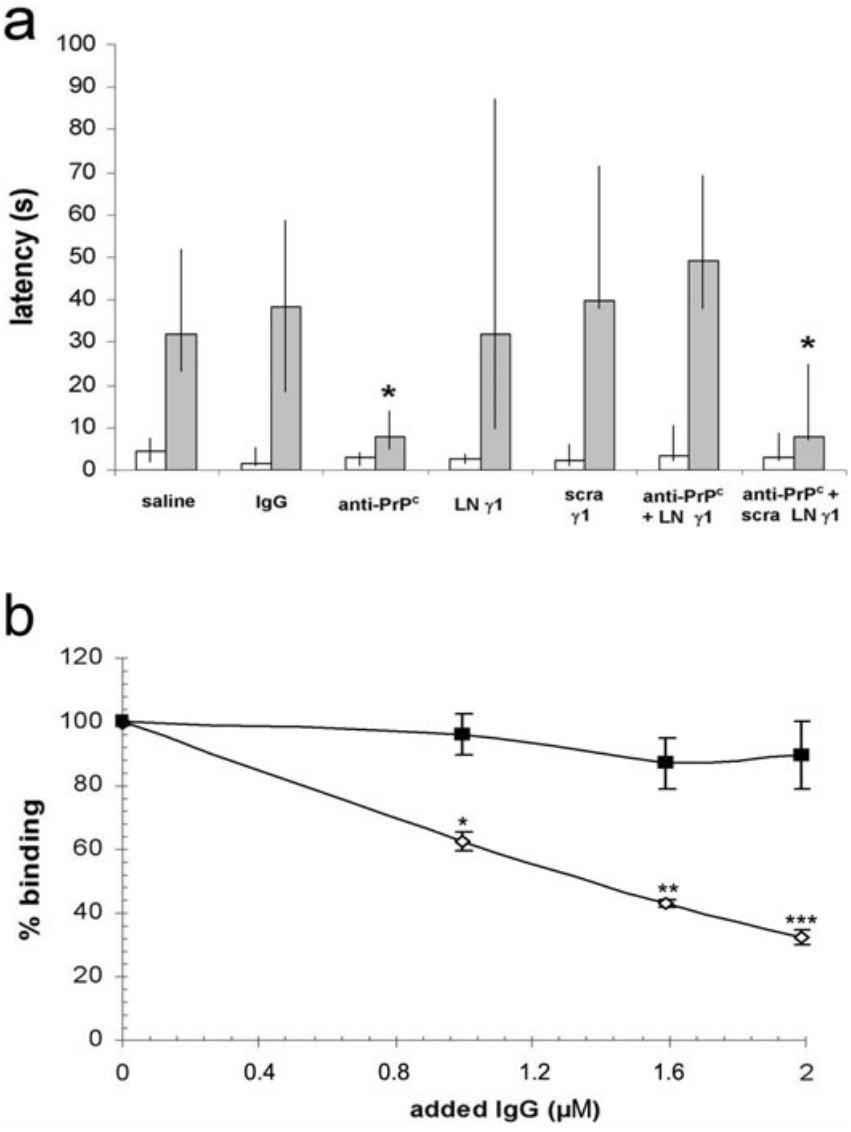

FIG. 4. PrP^c interaction with laminin is responsible for memory consolidation. (a) LN γ1 chain peptide reverses anti-PrP^c antibody inhibition on memory consolidation. Experiments were performed as in Fig. 1a. Latency to step down in the training trial was measured (white bars) and animals received intrahippocampal infusion of control IgG (1.8 μg/μL), anti-PrP^c IgG (1.8 μg/μL), LN γ1 chain peptide (0.08 μg/μL), LN γ1 chain scrambled peptide (0.08 μg/μL) [scra γ1], anti-PrP^c IgG (1.8 μg/μL) plus LN γ1 chain peptide (0.08 μg/μL) [anti-PrP^c + LN γ1] or anti-PrP^c IgG (1.8 μg/μL) plus LN γ1 scrambled peptide (0.08 μg/μL) [anti-PrP^c + scra LN γ1]. Memory retention was evaluated during a non-reinforced session carried 24 h post-training (grey bars). Statistical comparisons were performed using Kruskall–Wallis followed by Dunn's *post-hoc* test. *Post-training anti-PrP^c or anti-PrP^c + scra LN γ1 vs. post-training IgG, d.f. = 6, $P < 0.01$. (b) LN γ1 chain peptide binding to PrP^c is impaired by anti-PrP^c antibody. ^{125}I-LN γ1 chain peptide (1.2×10^{-6} M) was incubated in His_6-PrP^c (4 μg) adsorbed wells with increasing concentrations of control IgG (closed squares) or anti-PrP^c IgG (open diamonds). After extensive washing, radioactivity was measured. Results are expressed as a percentage of the PrP^c and ^{125}I-LN γ1 chain peptide binding. Data represent the mean ± SEM of three independent experiments and statistical analyses were performed by an independent samples Student's *t*-test. *Anti-PrP^c at 1 μM vs. IgG at 1 μM (d.f. = 4, $P < 0.05$); **anti-PrP^c at 1.6 μM vs. IgG at 1.6 μM (d.f. = 4, $P < 0.01$); ***anti-PrP^c at 2 μM vs. IgG at 2 μM (d.f. = 4, $P < 0.01$).

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expressed as relative levels compared with the value obtained for the treatment with control IgG (considered equal to 1). Data are expressed as mean ± SEM obtained for 7–8 animals per treatment and statistical analyses were performed by ANOVA followed by Tukey's HSD *post-hoc* test.

Results

To analyse the involvement of PrP^c and LN in memory consolidation, male Wistar rats were trained in a one-trial, step-down IA task, a highly validated, hippocampal-dependent learning task (Izquierdo & Medina, 1997). Immediately after IA training animals received bilateral intra-CA1 infusions (0.5 μL per side) of vehicle, control IgG or function-blocking anti-PrP^c and anti-LN antibodies. Memory was evaluated during a non-reinforced test session carried out 24 h post-training. When given immediately after training, both anti-PrP^c and anti-LN antibodies decreased test step-down latencies, whereas control IgG did not affect IA memory retention when compared with animals infused with saline (Fig. 1a). Rats that received anti-PrP^c or anti-LN antibodies immediately after training, normally acquired the avoidance response when submitted to a second training session 48 h after the first one (data not shown). In addition, when given into dorsal CA1 15 min before a 5-min open field session, anti-PrP^c antibody did not modify the latency to start locomotion, the number of crossings and rearings, or the quantity of fecal boluses (Fig. 1b–e). Anti-PrP^c and anti-LN antibodies did not alter the total number of entries or permanence into the open or into the closed arms of an elevated plus maze (Table 1). Thus, these experiments demonstrate specific impairing effects of antibody infusions on memory consolidation.

We have previously shown that PrP^c is a high-affinity ligand for LN and that the PrP^c-binding domain of LN comprises a decapeptide (RNIAEIIKDI) localized at the C-terminus of the LN γ1 chain (Graner *et al.*, 2000a). Indeed, binding experiments using ^{125}I-labeled LN γ1 chain peptide demonstrated its specific interaction with PrP^c (Fig. 2a). To evaluate whether PrP^c–LN interaction is important for IA memory consolidation we raised a polyclonal antibody specific for the LN γ1 chain C-terminal decapeptide. Western blot assays (Fig. 2b) demonstrated that this antibody recognizes the LN γ1 chain at around 250 kDa in purified LN 1 and in astrocyte and neuron cellular extracts. A band of around 150 kDa was also observed in both cell types, which

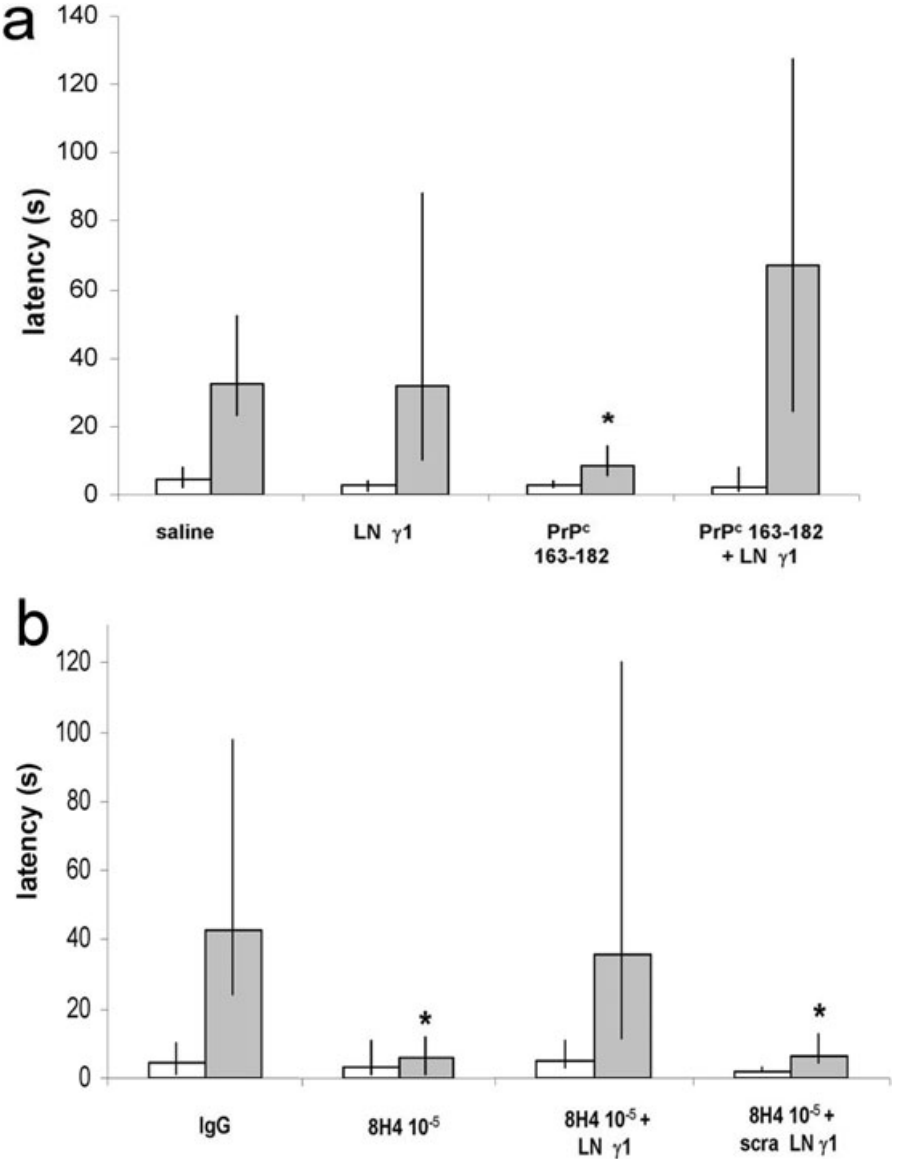

FIG. 5. PrP^c interaction with laminin is responsible for memory consolidation. (a) LN γ1 chain peptide reverses PrP^c peptide 163–182 inhibition. Experiments were performed as in Fig. 1a. Latency to step down in the training trial was measured (white bars) and animals received intrahippocampal infusion of LN γ1 chain peptide (RNIAEIIKDI) (0.08 μg/mL, 60 μM), PrP^c 163–182 (0.2 μg/mL, 60 μM) or LN γ1 chain peptide (0.08 μg/mL) plus PrP^c 163–182 (0.2 μg/mL). Memory retention was evaluated during a non-reinforced session carried out 24 h post-training (grey bars). Statistical comparisons were performed using Kruskall–Wallis followed by Dunn's *post-hoc* test. *Post-training PrP^c 163–182 vs. saline, d.f. = 3, $P < 0.05$. (b) LN γ1 peptide reverses anti-PrP^c antibody, 8H4, inhibition. Experiments were performed as in Fig. 1a. Latency to step down in the training trial was measured (white bars) and animals received infusion of mouse non-immune IgG (10^{-3} μg/μL), anti-PrP^c 8H4 (10^{-5} μg/μL) plus LN γ1 chain peptide (0.08 μg/μL) or anti-PrP^c 8H4 (10^{-5} μg/μL) plus LN γ1 chain scrambled peptide (0.08 μg/μL). Memory retention was evaluated during a non-reinforced session carried out 24 h post-training (grey bars). Statistical comparisons were performed using Kruskall–Wallis followed by Dunn's *post-hoc* test. *Post-training 8H4 at 10^{-5} μg/μL or 10^{-5} μg/μL + scra LN γ1 vs. IgG, d.f. = 3, $P < 0.01$.

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probably represents a laminin degradation product. When given into CA1 immediately after IA training this antibody dose-dependently hindered memory consolidation. Control IgG had no effect on IA memory retention when compared with the saline-infused group (Fig. 1a), even given at a concentration 100 times higher than that of anti-LN γ1 chain peptide (Fig. 2c).

To analyse further the role played by PrP^c–LN interaction in memory consolidation, we first mapped the LN γ1-binding site at the PrP^c molecule and then determined the effect of peptides derived from this domain on IA memory retention. Using an *in vitro* competition assay, we found that only two overlapping peptides ($PrP^c_{163-182}$ and $PrP^c_{173-192}$) out of a series of 20-mers covering the entire PrP^c molecule inhibited the interaction between ^{125}I-labeled LN γ1 peptide and full-length recombinant PrP^c (Fig. 3a). Importantly, $PrP^c_{163-182}$ and $PrP^c_{173-192}$ but not PrP^c_{73-92}, a 20-mer unable to affect ^{125}I-LN γ1-PrP^c binding, impaired IA memory retention when given into dorsal CA1 immediately after training (Fig. 3b). Moreover, intra-CA1 infusion of antibody 8H4 (Cui *et al*., 2003), a monoclonal antibody against $PrP^c_{175-185}$ (i.e. a linear epitope within the putative LN γ1-binding site at PrP^c), also blocked memory consolidation (Fig. 3c). Conversely, an antibody against PrP^c_{34-45}, 8B4 (Cui *et al*., 2003), an epitope outside the presumed LN γ1-binding domain, had no effect on memory retention (Fig. 3c) even at a concentration 1000 times higher than that used for 8H4 antibody.

It was still necessary, however, to demonstrate conclusively that the involvement of PrP^c and LN in memory consolidation depended on their interaction. Although a positive effect was predictable for LN γ1 chain peptide, we found that the intra-CA1 infusion of this peptide (0.08 μg/μL) did not affect IA memory consolidation (Fig. 4a). This result could be due to its ligand (PrP^c) or signaling saturation by the LN already present in the system. Nonetheless, co-infusion of the LN γ1 chain peptide was able to prevent the amnesic effect of intrahippocampal anti-PrP^c antibody (Fig. 4a). The intra-CA1 infusion of a control scrambled γ1 chain peptide did not affect IA memory retention or the amnesic effect produced by the intrahippocampal infusion of anti-PrP^c.

As the antibody used in these experiments was a polyclonal against the entire PrP^c molecule (the same utilized in the experiment depicted in Fig. 1), we carried out competition experiments to demonstrate that this antibody is able to dissociate PrP^c-LN γ1 chain peptide binding (Fig. 4b).

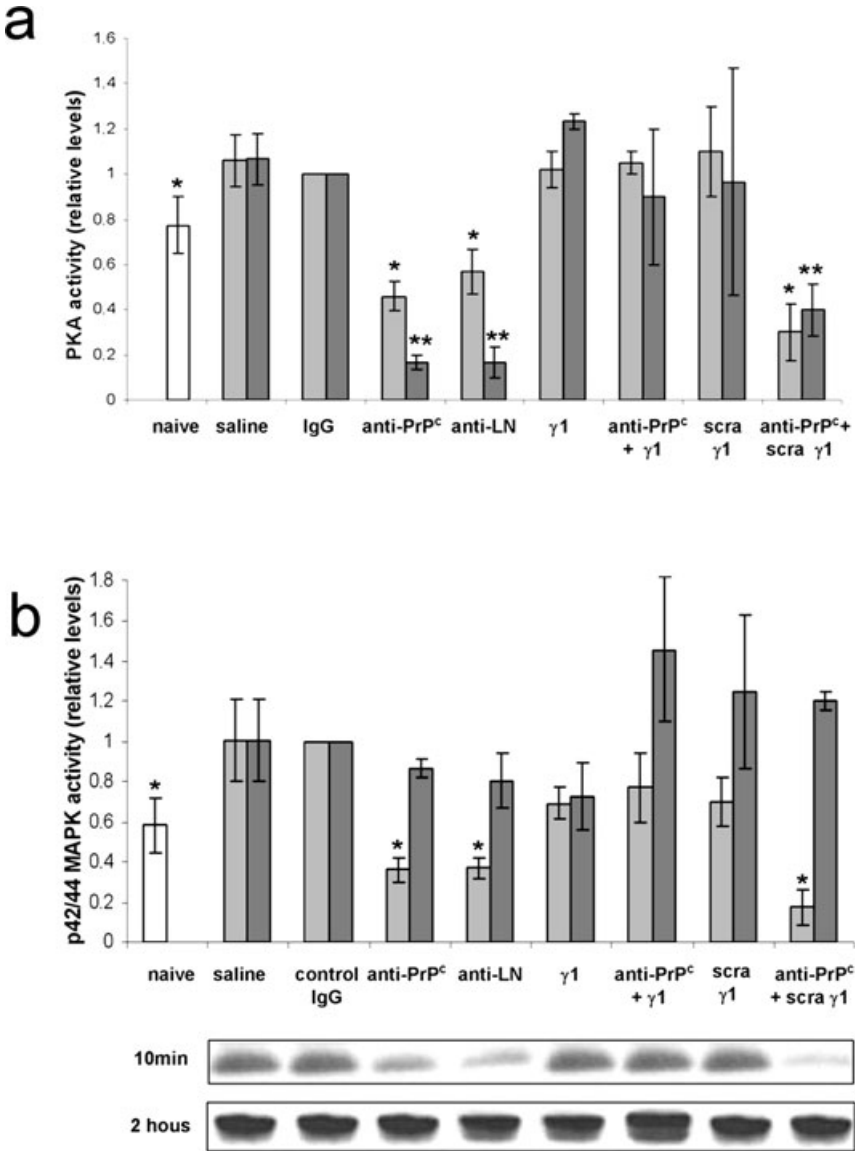

FIG. 6. PrP^c–LN interaction triggers signal through the PKA and ERK1/2. Rats received immediately post-training bilateral hippocampal infusions of: saline, control rabbit IgG (1.8 μg/μL), anti-PrP^c IgG (1.8 μg/μL), anti-LN IgG (0.18 μg/μL), LN γ1 chain peptide (0.08 μg/μL), LN γ1 chain scrambled peptide (0.08 μg/μL) [scra γ1], anti-PrP^c IgG (1.8 μg/μL) plus LN γ1 chain peptide (0.08 μg/μL) [anti-PrP^c + γ 1] and anti-PrP^c IgG (1.8 μg/μL) plus LN γ1 chain scrambled peptide (0.08 μg/μL) [anti-PrP^c + scra γ1]. Naive animals (white bars), or those infused [10 min (light grey bars) or 2 h after training (dark grey bars)] were killed and hippocampi nuclear extracts used for enzymatic assays. PKA (a) or ERK1/2 (b) activities were determined using assay systems according to the manufacturer's instructions. Enzymes activities of each treatment were expressed as relative levels compared with the value from treatment with non-immune IgG (considered to be equal to 1). Figures below graphic (b) show autoradiograms obtained in ERK1/2 activity assays. Results were represented as means ± SEM and statistical analysis were performed by ANOVA followed by Tukey's HSD *post-hoc* test. *10 min after training anti-PrP^c or anti-LN or anti-PrP^c + scra γ1 or naïve animals vs. 10 min after training IgG (d.f. = 8, $P < 0.05$). **2 h after training anti-PrP^c or anti-LN or anti-PrP^c + scra γ1 vs. 2 h after training IgG (d.f. = 8, $P < 0.05$).

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Additionally, co-infusion of the LN γ1 peptide abolished the amnesic effect induced by the post-training intra-CA1 infusion of $PrP^{c}_{163-182}$ peptide (Fig. 5a). Memory consolidation was also rescued when the LN γ1 chain peptide, but not the scrambled one, was co-infused with antibody 8H4 (Fig. 5b), which recognizes the LN binding site at the PrP^{c} molecule and also blocks memory retention. Thus, these data indicate that the effects of 8H4 anti-PrP^{c} antibody and $PrP^{c}_{163-182}$ peptide in memory consolidation are due to impairment of the PrP^{c}–LN interaction.

We have previously shown that the early activation of hippocampal PKA and ERK1/2-mediated signaling pathways is required for consolidation of IA memory (Bernabeu *et al*., 1997; Alonso *et al*., 2002; Rossato *et al*., 2004). By contrast, it is known that PrP^{c} can induce the activation of PKA and ERK1/2 in several experimental systems (Chiarini *et al*., 2002; Monnet *et al*., 2004; Lopes *et al*., 2005; Krebs *et al*., 2006), and neuronal plasticity (neuritogenesis) induced by PrP^{c}-LN γ1 peptide is dependent at least on ERK1/2 phosphorylation (F. H. Beraldo and V. R. Martins, unpublished data). Therefore, we decided to determine whether the amnesia caused by interfering with the PrP^{c}–LN association was accompanied by any modification in the learning-induced up-regulation of hippocampal PKA and ERK1/2.

Rats with infusion cannulae aimed to the CA1 region of the dorsal hippocampus were trained in the IA task and immediately after training received intra-CA1 infusions of saline, anti-PrP^{c}, anti-LN, anti-PrP^{c} antibody plus the LN γ1 chain peptide or anti-PrP^{c} antibody plus a scrambled γ1 peptide. Animals were killed 10 min or 2 h after training and hippocampal nuclear extracts used to measure PKA and ERK1/2 activities. As can be seen in Fig. 6, trained animals infused with saline showed higher PKA (Fig. 6a) and ERK1/2 (Fig. 6b) activities than naïve animals, confirming previous data from others and also from our group (Bernabeu *et al*., 1997; Alonso *et al*., 2002; Rossato *et al*., 2004). The IA-induced activation of nuclear PKA (Fig. 6a) and ERK1/2 (Fig. 6b) was blocked by the immediate post-training administration of anti-PrP^{c} or anti-LN antibodies. Importantly, the blocking effect of anti-PrP^{c} antibody was completely abrogated by co-infusion of the LN γ1 chain peptide but not by the scrambled γ1 peptide.

Discussion

Our experiments show that intra-CA1 infusion of function-blocking anti-PrP^{c} and anti-LN antibodies immediately after training hindered retention of IA long-term memory. We also found that when submitted to a second training session, those same animals normally acquired the avoidance response and did so as if they had never been trained before (data not shown). Additionally, in spite of previous evidence that mice devoid of PrP^{c} display altered locomotor activity (Roesler *et al*., 1999) the control experiments carried out in the present study showed that the memory-impairing effects of intrahippocampal infusions could not be attributed to alterations in locomotion or exploratory behavior. The discrepancies between pharmacological models observed in this study and genetic models used previously (Roesler *et al*., 1999) might be related to compensatory changes such as up-regulation of alternative signaling pathways in PrP^{c} null mice (Bueler *et al*., 1992).

Therefore, the IA experiments together with plus maze and open field results suggest that anti-LN and anti-PrP^{c} antibodies do not produce any insult on hippocampal functionality. Moreover, they indicate that their amnesic effect is due to an action on the consolidation process and is not caused by an impairment of exploratory and locomotor activities. Furthermore, the data showing that only those reagents which specifically block PrP^{c}-LN binding are able to hinder IA memory consolidation and strongly indicate that this process indeed requires interaction between these proteins. Thus, PrP^{c} may be added to the list of putative LN receptors involved in memory consolidation.

During early stages of memory formation some proteases such as metalloprotease-9 and tissue plasminogen activator (tPA) can be activated (Qian *et al*., 1993; Nagy *et al*., 2006). The proteolytic cleavage of their substrates is complex and includes changes in physical constraints of the pericellular milieu, liberation of sequestered molecules and exposure of latent bioactive peptides (Nagase & Woessner, 1999). It is well known that laminin is a substrate for these proteases (Chen & Strickland, 1997; Gu *et al*., 2005) and it might be possible that controlled laminin digestion alters the exposure of specific domains such as that containing the γ1 peptide, increasing its affinity to PrP^{c}. It is also possible that active peptides are generated by protease activity. Thus, although PrP^{c} and laminin are constitutively expressed in the hippocampus, their high affinity interaction and signaling may be dependent on the exposition/conformation of specific domains. As a result, the effect mediated by PrP^{c} and laminin antibodies and peptides must be the blockage of interacting sites within the molecules.

Integrins, the classical LN receptors (Giancotti, 2000), participate in long-term potentiation (Lynch, 1998; Kramar *et al*., 2002; Kramar & Lynch, 2003) and antibodies against integrin-associated protein can block memory retention in rats (Chang *et al*., 2001). In fact, it has been shown that mice with reduced expression of α3, α5 and α8 integrins are defective in hippocampal long-term potentiation and spatial memory (Chan *et al*., 2003). However, although it is known that integrins may associate with the N-terminal domain of LN γ1 chain (Ponce *et al*., 2001), it has been demonstrated that PrP^{c} is the unique receptor for the C-terminus domain of the LN γ1 chain (Graner *et al*., 2000a). Moreover, the LN γ1 peptide is unable to induce neuritogenesis in PrP^{c} null neurons (Graner *et al*., 2000a) which do express integrins, strongly indicating that integrins do not bind this LN γ1 peptide or compete with PrP^{c} for this interaction.

The participation of hippocampal PKA and ERK1/2 in LTM consolidation is well documented (Yin *et al*., 1994; Martin *et al*., 1997), suggesting that inhibition of the learning-induced activation of these kinases may be responsible for the amnesic effect of PrP^{c} and LN blocking agents. This hypothesis is reinforced by the fact that the LN γ1 chain peptide reverses the amnesic effect of anti-PrP^{c} antibodies. These results suggest that PrP^{c} can indeed participate in learning and memory processes through regulation of key signaling pathways. In this respect, it has been demonstrated that ERK1/2 are targets of PrP^{c} signaling in neuronal and non-neuronal cells (Schneider *et al*., 2003; Chen *et al*., 2003). Additionally, the PrP^{c}–LN interaction induces neuronal adhesion and differentiation (Graner *et al*., 2000a,b) and LN γ1 peptide added to hippocampal cultures of wild-type neurons but not in PrP^{c} null neurons induces neuritogenesis, a plasticity phenomenon largely related to memory formation, in a ERK1/2-dependent way (F. H. Beraldo and V. R. Martins, unpublished results). Furthermore, another essential enzyme to memory consolidation, PKA, has also been shown to be activated upon PrP^{c} signaling (Chiarini *et al*., 2002; Lopes *et al*., 2005).

The approach presented here is a suitable alternative to establish the role of PrP^{c} in cognition avoiding misinterpretation related to compensatory mechanisms, genetic manipulation and background of PrP^{c} gene (*Prnp*) ablated mice. Previous work has shown that memory is not disrupted in mice devoid of *Prnp*, which can be explained by the obvious redundancy that must exist in order to

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preserve such an important phenotype (Bueler *et al*., 1992). Alterations in essential cellular signaling pathways such as higher PKA and ERK1/2 activities have been described in two lines of *Prnp* ablated animals with different genetic backgrounds (Brown *et al*., 2002; Chiarini *et al*., 2002). As both enzyme activities are essential for memory consolidation (Abel *et al*., 1997; Izquierdo & Medina, 1997; Atkins *et al*., 1998), it is plausible to speculate that their altered pattern contributes to compensatory mechanisms in these mice.

In conclusion, our results indicate that hippocampal PrP^{c} and LN are important for the consolidation of fear memory and suggest that the role of these proteins during memory processing involves their direct interaction and the activation of PKA and ERK1/2-controlled intracellular cascades.

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Abbreviations

ERK1/2, extracellular regulated kinase; IA, inhibitory avoidance; LN, laminin; LTM, long-term memory; PKA, cyclic AMP-dependent protein kinase; *Prnp*, cellular prion protein gene; PrP^{c}, cellular prion protein; PrP^{sc}, scrapie prion protein.

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