The interaction between prion protein and laminin modulates memory consolidation

Adriana S. Coitinho,1* Adriana R. O. Freitas,2* Marilene H. Lopes,2,6* Glaucia N. M. Hajj,2* Rafael Roesler,3 Roger Walz,4 Janine I. Rossato,5 Martin Cammarota,5 Ivan Izquierdo,5 Vilma R. Martins2,6* and Ricardo R. Brentani6,7

1Centro Universitário Feevale, Instituto de Ciências da Saúde, RS 239, 2755, 93352-000, Novo Hamburgo, RS, Brazil
2Ludwig Institute for Cancer Research, São Paulo Branch, Rua Prof. Antônio Prudente 109/4A, 01509-010, São Paulo, SP, Brazil
3Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde, UFRGS, Rua Sarmento Leite, 500, 90046-900 Porto Alegre, RS, Brazil
4Centro de Cirurgia de Epilepsia do Estado de Santa Catarina, Hospital Governador Celso Ramos and Departamento de Clínica Médica-Hospital Universitário, UFSC, Florianópolis, SC, Brazil
5Centro de Memória, Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica, Porto Alegre, RS, Brazil
6Centro de Tratamento e Pesquisa Hospital do Câncer Rua Prof. Antônio Prudente 109/4A, 01509-010, São Paulo, SP, Brazil
7Departamento de Radiologia, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil

Keywords: cellular prion, hippocampus, laminin, learning, memory, rat

Abstract
Cellular prion protein (PrPc) has a pivotal role in prion diseases. PrPc 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 PrPc and LN plays a role in rat memory formation. We found that post-training intrahippocampal infusion of PrPc-derived peptides that contain the LN binding site (inhibitory avoidance memory retention. The amnesic effect of anti-PrPc antibodies and for memory consolidation. In addition, co-infusion of a LN regulator of signaling pathways needed for consolidation of inhibitory avoidance memory. Our findings show that, through its hippocampal cAMP-dependent protein kinase A (PKA) and extracellular regulated kinase (ERK1/2) signaling pathways. The Authors (2006). Journal Compilation © Federation of European Neuroscience Societies and Blackwell Publishing Ltd

Introduction
Cellular prion protein (PrPc) is a cell surface, glycosylphosphatidylinositol-annealed protein that is abundantly expressed in neurons. Evidence indicates that conversion of PrPc into its abnormal conformer, the scrapie prion protein (PrPsc), causes transmissible spongiform encephalopathies (Prusiner et al., 1998). PrPc has been associated with regulation of ion transport, synaptic transmission and neurogenesis, suggesting a role in neuronal plasticity (Reviewed by Martins & Brentani, 2002). In fact, the participation of PrPc in learning and memory processes 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 PrPc 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).

Laminins (LNs) are components of the extracellular matrix formed by heterotrimeric molecules composed by α, β and γ chains (Colognato & Yurchenco, 2000). LN-10 (α3β1γ1) 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 PrPc is a receptor for a decapptide (RNAIIEIKDI) at the C-terminus of the LN γ1 chain (Graner et al., 2000a). Thus, neurogenesis by hippocampal cultures in the presence of this peptide was inhibited by anti-PrPc antibodies. Furthermore, no neurogenesis was elicited by LN-γ1 chain peptide in neurons obtained from PrPc-Prnp−/− null mice, indicating that a PrPc-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 systems.
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).

Here, we demonstrate the relevance of PrPc–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 PrPc–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 PrPc or anti-LN on plus-maze performance

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>Open arms</td>
<td>5.7 ± 0.8</td>
</tr>
<tr>
<td>Number of entries</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Permanency (s)</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Closed arms</td>
<td>7.6 ± 1.3</td>
</tr>
<tr>
<td>Number of entries</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td>Permanency (s)</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>

Animals received IgG, anti-PrPc 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.
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...
number of line crossings and the number of rearsings 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-PrPc, anti-LN or non-immune serum into the CA1 region of the dorsal hippocampus. Data are shown as mean ± SEM number of crossings, rearsings, 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-PrPc IgG was raised in rabbit against the recombinant GST-PrPc 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 PrPc 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 PrPc 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 (RNIAEIKIDI) was produced by Bethyl Inc. (TX, USA). PrPc peptides, laminin γ1 chain derived peptide (RNIAEIKIDI) and laminin γ1 scrambled peptide (IRANIEIKID) were from Neosystem (Strasbourg, France).

Expression and purification of recombinant PrPc
The expression vector containing the cDNA fragment encoding amino acids 23–231 of the mouse PrPc protein was kindly provided by Dr Ralph Zahn (Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, Switzerland). His6-PrPc expression and purification were performed as previously described (Zanata et al., 2002).

PrPc–LN γ1 peptide binding and competition assays
His6-PrPc (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 125I-LN γ1 chain peptide (RNIAEIKIDI) linked to BSA (labeled as described by Chiarini et al., 2002) with specific activity of $7 \times 10^5$ 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, His6-PrPc (4 µg) was incubated with 125I-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. PrPc domain that interacts with LN is related to memory consolidation.](Image)

(a) Mapping LN binding site domain using a competition assay with PrPc peptides. Peptides (3.8 × 10^{-5} M) covering the mouse PrPc (23–231) sequence were pre-incubated with 1.2 × 10^{-6} M 125I-LN γ1 chain peptide followed by incubation in His6-PrPc adsorbed wells. After extensive washing, radioactivity was measured. Total His6-PrPc and 125I-LN γ1 chain peptide binding was set as 100% and the results expressed as the relative percentage of binding produced with 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) PrPc 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 PrPc peptides 73–92, 163–182 or 173–192 (0.2 µg/µL). 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 PrPc 163–183 or PrPc 173–192 vs. post-training saline, d.f. = 3, $P < 0.01$. (c) Monoclonal antibody against the PrPc 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, $P < 0.01$. © The Authors (2006). Journal Compilation © Federation of European Neuroscience Societies and Blackwell Publishing Ltd European Journal of Neuroscience, 24, 3255–3264
Competition assays were performed using PrP\(^\text{c}\) synthetic peptides and antibodies. Synthetic mouse PrP\(^\text{c}\) peptides (Zanata et al., 2002), 3.8 × 10\(^{-5}\) M, anti-PrP\(^\text{c}\) IgG or non-immune serum IgG were pre-incubated with 1.2 × 10\(^{-6}\) M \(^{125}\text{I-LN}\) γ1 chain peptide-BSA for 3 h at room temperature. Then, the reagents were added to the wells containing 4 μg adsorbed His\(_\text{6-PrP}\) and incubated for 16 h at 4 °C. After extensive washing, incorporated radioactivity was determined using a gamma counter. The PrP\(^\text{c}\) \(^{125}\text{I-LN}\) 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\(^\text{c}\) 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\(^\text{c}\) 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**

Naive 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 antiphospho 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 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\(^\text{c}\) or anti-PrP\(^\text{c}\) + scra LN γ1 vs. post-training IgG, d.f. = 6, P < 0.01. (b) LN γ1 chain peptide binding to PrP\(^\text{c}\) is impaired by anti-PrP\(^\text{c}\) antibody. \(^{125}\text{I-LN}\) γ1 chain peptide (1.2 × 10\(^{-6}\) M) was incubated in His\(_\text{6-PrP}\) (4 μg) adsorbed wells with increasing concentrations of control IgG (closed squares) or anti-PrP\(^\text{c}\) IgG (open diamonds). After extensive washing, radioactivity was measured. Results were expressed as a percentage of the PrP\(^\text{c}\) and \(^{125}\text{I-LN}\) 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\(^\text{c}\) at 1 μM vs. IgG at 1 μM (d.f. = 4, P < 0.05); **anti-PrP\(^\text{c}\) at 1.6 μM vs. IgG at 1.6 μM (d.f. = 4, P < 0.01); ***anti-PrP\(^\text{c}\) at 2 μM vs. IgG at 2 μM (d.f. = 4, P < 0.01).
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 PrPc 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-PrPc 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-PrPc 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-PrPc 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-PrPc antibody did not modify the latency to start locomotion, the number of crossings and rearings, or the quantity of fecal boluses (Fig. 1b–c). Anti-PrPc 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 PrPc is a high-affinity ligand for LN and that the PrPc-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 125I-labeled LN γ1 chain peptide demonstrated its specific interaction with PrPc (Fig. 2a). To evaluate whether PrPc–LN interaction is important for IA memory consolidation we raised a polyclonal antibody specific for the LN γ1 chain C-terminal decapetide. 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

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 PrPc–LN interaction in memory consolidation, we first mapped the LN γ1-binding site at the PrPc 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 (PrPc173–192 and PrPc163–182) out of a series of 20-mers covering the entire PrPc molecule inhibited the interaction between 125I-labeled LN γ1 peptide and full-length recombinant PrPc (Fig. 3a). Importantly, PrPc163–182 and PrPc173–192 but not PrPc173–192, a 20-mer unable to affect 125I-LN γ1-PrPc 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 PrPc175–185 (i.e. a linear epitope within the putative LN γ1-binding site at PrPc), also blocked memory consolidation (Fig. 3c). Conversely, an antibody against PrPc134–145, 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 PrPc 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 (PrPc) 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-PrPc 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-PrPc.

As the antibody used in these experiments was a polyclonal against the entire PrPc 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 PrPc–LN γ1 chain peptide binding (Fig. 4b).

**Fig. 6.** PrPc–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-PrPc 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-PrPc IgG (1.8 μg/μL) plus LN γ1 chain peptide (0.08 μg/μL) [anti-PrPc + γ1] and anti-PrPc IgG (1.8 μg/μL) plus LN γ1 chain scrambled peptide (0.08 μg/μL) [anti-PrPc + 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-PrPc or anti-LN or anti-PrPc + scra γ1 or naive animals vs. 10 min after training IgG (d.f. = 8, P < 0.05). **2 h after training anti-PrPc or anti-LN or anti-PrPc + scra γ1 vs. 2 h after training IgG (d.f. = 8, P < 0.05).

© The Authors (2006). Journal Compilation © Federation of European Neuroscience Societies and Blackwell Publishing Ltd

*European Journal of Neuroscience, 24, 3255–3264*
Additionally, co-infusion of the LN γ1 peptide abolished the amnesic effect induced by the post-training intra-CA1 infusion of PrPc-LN γ1 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 molecule and also blocks memory retention. Thus, these data indicate that the effects of 8H4 anti-PrP antibody and PrPc-LN γ1 peptide in memory consolidation are due to impairment of the PrPc-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 PrPc 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 (neurotogenesis) induced by PrPc-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 PrPc–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 learning-induced up-regulation of hippocampal 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-PrPc or anti-LN antibodies. Importantly, the blocking effect of anti-PrPc 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-PrPc 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 PrPc 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 PrPc null mice (Bueler et al., 1992).

Therefore, the IA experiments together with plus maze and open field results suggest that anti-LN and anti-PrPc 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 PrPc-LN binding are able to hinder IA memory consolidation and strongly indicate that this process indeed requires interaction between these proteins. Thus, PrPc 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 that containing the γ1 peptide, increasing its affinity to PrPc. It is also possible that active peptides are generated by protease activity. Thus, although PrPc 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 PrPc 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 PrPc 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 neurotogenesis in PrPc null neurons (Graner et al., 2000a) which do express integrins, strongly indicating that integrins do not bind this LN γ1 peptide or compete with PrPc 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 PrPc and LN blocking agents. This hypothesis is reinforced by the fact that the LN γ1 chain peptide reverses the amnesic effect of anti-PrPc antibodies. These results suggest that PrPc 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 PrPc signaling in neuronal and non-neuronal cells (Schneider et al., 2003; Chen et al., 2003). Additionally, the PrPc–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 PrPc null neurons induces neurotogenesis, a plasticity phenomenon largely related to memory formation, in an 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 PrPc signaling (Chiarini et al., 2002; Lopes et al., 2005).

The approach presented here is a suitable alternative to establish the role of PrPc in cognition avoiding misinterpretation related to compensatory mechanisms, genetic manipulation and background of PrPc 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
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; Chiariini 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 PrPc 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.

Acknowledgements

We thank Dr Man-Sun Sy (Case Western Reserve University, USA) for providing the monoclonal antibodies 8H4 and 8B4 and Dr Ralph Zahn (Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, Switzerland) for the PrPc expressing vector. This work was supported by grants from FAPESP (99/07124-8, 03/13189-2), CNpq and PRONEX. A.R.O.F., M.H.L. and G.N.M.H. are supported by FAPESP.

References


Cellular prion and memory consolidation 3263


