PLEIOTROPHIN INHIBITS HIPPOCAMPAL LONG TERM POTENTIATION:
A ROLE OF PLEIOTROPHIN IN LEARNING AND MEMORY

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ABSTRACT

Pleiotrophin (PTN) is a growth factor that has been shown to be involved in hippocampal synaptic plasticity and learning. To further understand the involvement of PTN in memory processes, we performed in vitro electrophysiological studies in PTN-stimulated CA1 from rat hippocampal slices combined with the behavioural testing of PTN deficient (PTN -/-) mice. We found that PTN inhibited hippocampal Long Term Potentiation (LTP) induced by high-frequency stimulation (HFS) consisted in three trains of 100 Hz separated by 20 s. To test the possibility that PTN might be involved in behavioural memory processes, we tested the learning behaviour of PTN -/- mice using the Y-maze test. We did not observe significant differences in recognition memory between PTN -/- and Wild Type (WT) mice when a 30 min-ITI (interval intertrial) was used in the Y-maze test. However, whereas WT mice showed disruption of recognition memory using a 60 min-ITI, PTN -/- mice maintained the recognition memory. The data demonstrate that PTN inhibits hippocampal LTP in vitro and might play a role in memory processes in vivo.

Keywords: LTP, Y-maze, receptor protein tyrosine phosphatase (RPTP) β/ζ, p190 RhoGAP, synaptic plasticity, learning and memory.
INTRODUCTION

Pleiotrophin (PTN), also known as heparin-binding growth-associated molecule (HB-GAM) (Rauvala, 1989), is a secreted, highly conserved 136-amino acid growth factor (Milner et al., 1989; Li et al., 1990) that is expressed during development and early differentiation of neurons and glia within the central nervous system (Deuel et al., 2002). In the adult brain, PTN expression is restricted to limited cell populations such as striatal interneurons (Taravini et al., 2005) and CA1 hippocampal neurons (Lauri et al., 1996) in which is expressed in an activity dependent manner (Wanaka et al., 1993).

The mechanism of action of PTN is unique. Pleiotrophin signals through binding to the Receptor Protein Tyrosine Phosphatase (RPTP)β/ζ (Meng et al., 2000) inactivating its phosphatase activity leading to increased levels of tyrosine phosphorylation of the substrates of RPTPβ/ζ including β-catenin (Meng et al., 2000), GIT1/Cat-1 (Kawachi et al., 2001), Fyn (Pariser et al., 2005a), β-adducin (Pariser et al., 2005b; Pariser et al., 2005c), p190 RhoGAP (Tamura et al., 2006) and Anaplastic lymphoma kinase (ALK) (Perez-Pinera et al., 2007). To the best of our knowledge, binding and ligand-induced effects through any Receptor-type PTPs have been only demonstrated in the case of PTN and RPTPβ/ζ.

Pleiotrophin has been previously found to modulate Long-term potentiation (LTP) suggesting an important role of PTN in hippocampal plasticity (Pavlov et al., 2002). Long-term potentiation is the best studied form of synaptic plasticity in the hippocampus and it is considered to be one of the cellular bases of learning and memory (Bliss and Collingridge, 1993; Lynch, 2004). The involvement of LTP-like mechanisms in learning and memory has been well documented (Eichenbaum and Otto, 1993; Lynch, 2004). Pleiotrophin mRNA expression is induced after high frequency stimulation (HFS)-induced LTP (Lauri et al., 1996). It was found that PTN injection
into the dendritic area of CA1 pyramidal neurons inhibited tetanus-induced LTP in rat hippocampal slices, an effect that was limited to early, synapse-specific stages of LTP induction (Lauri et al., 1998). In that study, it was not clear whether this effect was merely due to the inhibition of the function of endogenous protein expressed in the pyramidal neurons or the result of specific inhibitory effect on LTP itself.

To establish the role of PTN in LTP and its relationship with behavioral tasks, we have now tested the effects of PTN in hippocampal slices using a different experimental approach. We tested the effects of a low concentration of PTN perfusion on HFS-induced LTP in rat hippocampal slices. In addition, we analyzed the behavioural effects of PTN in vivo by testing spatial memory of PTN genetically deficient (PTN -/-) mice using the Y maze test.
MATERIAL AND METHODS

Animals

PTN -/- mice were generated on a 129/Ola x C57BL/6J background by deleting exons 2 to 4 as previously described (Amet et al., 2001; Ezquerra et al., 2004). In these studies we used 7-9 weeks old (20-25 g) male PTN -/- and wild type (WT, +/-) mice resulting from intercrosses between 129/Ola x C57BL/6J.

For electrophysiological experiments naive adult male Sprague-Dawley rats (200-220 g; San Pablo CEU University breeding colony) were used.

All the animals used in this study were maintained according to European Union Laboratory Animal Care Rules (86/609/ECC directive) and the protocols were approved by the Animal Research Committee of USP-CEU.

Electrophysiology

Our purpose for these experiments was to assess the effects of PTN (Sigma, Spain) addition to perfusion solution on hippocampal LTP, assay that, to the best of our knowledge, had not been studied before. To be able to compare our results with previous reports showing the effects of PTN on LTP (Lauri et al., 1998), we used adult male Sprague-Dawley rats that were sacrificed by decapitation. Their brains were removed immediately and placed in bubbled (95% O₂ and 5% CO₂) and ice-cold Krebs–Ringer bicarbonate (KRB) solution containing (in mM): 109 NaCl, 2.5 KCl, 1 KH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂, 26.2 NaHCO₃ and 11 glucose. As described previously (del Olmo et al., 2000; del Olmo et al., 2003), transverse slices (400 μm) of each hippocampus were cut with a manual tissue chopper and placed in a humidified interface chamber at room temperature (20–25 °C). After a 2 h incubation period, the slices were transferred to the submersion recording chamber which was continuously
perfused with standard KRB solution at a rate of 1.8–2 ml/min. Field excitatory postsynaptic potentials (fEPSPs) were recorded for 100 min in the CA1 stratum radiatum with tungsten electrodes (1 MΩ) and evoked by stimulating Schaffer collateral–commissural fibres with biphasic electrical pulses (30–70 μA; 100 μs; 0.033 or 0.066 Hz) delivered through bipolar tungsten insulated microelectrodes (0.5 MΩ). The recording electrode was connected to an AI-402 amplifier (Axon Instruments, USA) plugged to a CyberAmp 380 signal conditioner (Axon Instruments, USA). Electrical pulses were supplied by a Master 8 pulse generator (AMPI, Israel). Evoked responses were digitalized at 25–50 Hz using a Digidata 1322A (Axon Instruments, USA) and stored on a Pentium IV IBM compatible computer using pCLAMP 9.0 software (Axon Instruments, USA).

The Schaffer collateral axons were stimulated every 15 s. Stimulus strength was adjusted to evoke a response of 40-50% of maximal fEPSP slope. After obtaining stable synaptic responses for at least 20 min (baseline period), the pathway was tetanized with three 100 Hz pulses for 1 s and of 100 μs duration every 20 sec (high-frequency stimulation, HFS). The synaptic strength was assessed by measuring the initial slope of the fEPSP, as determined by the pCLAMP 9.0 software. The data were normalized according to the mean values of the responses obtained from each animal during the 20 min baseline period. A single slice from each animal was considered as n=1. All electrophysiological experiments were carried out at 31–32 °C and all the chemicals used in these studies were obtained from Sigma (Spain).

Pleiotrophin (3 μM) or Phosphate Buffered Saline (vehicle, control) were added to KRB solution and applied via the perfusion line for 10 min and 10 min before tetanization. A 3 μM concentration of PTN has been demonstrated to be effective in different in vitro studies (Gramage et al., 2008).
The average values of the initial slope of the fEPSP were analyzed by a repeated measure ANOVA. Unpaired t-tests were performed to determine specific group differences in the average fEPSP measured during 5 consecutive minutes at different times of the recording assay. In all cases, statistical differences were considered significant if the probability of error was less than 5%. All calculations were performed using the SPSS statistical package 15.0 version.

Y-maze testing

A two-trial memory task, based on a free-choice exploration paradigm in a Y-maze was used to study recognition processes in mice in response to novelty and working memory. Exploration was measured with a short (2 min) intertrial interval (ITI) between acquisition and retrieval, while memory was examined with longer intervals (30 min and 1 h). During the first trial (acquisition), the animal is allowed to visit two arms of a Y-maze, the third being blocked with a door. During the second trial (retrieval), the door is opened, and the animal has free access to all three arms. Discrimination of novelty versus familiarity can then be studied by comparing exploration of the three arms. Memory can be tested by evaluating the influence of various intertrial intervals (ITIs) on recognition performance.

Mice (WT, n = 13; PTN -/-, n = 14) were housed 3 to 4 per cage in a temperature (22°C) and humidity (60%) controlled room on a 12-12 h light-dark (light 06:00–18:00) schedule. The mice were allowed to acclimate to the facilities for a minimum of 1 week before the beginning of the studies and had free access to food and water throughout the experiments. The mice were 7-9 weeks old (20-25 g) when tested. All the experiments were conducted during the light phase.

Response to novelty was tested in a Y-maze, using a two-trial procedure. The apparatus was constructed of black plastic with three arms each 34 cm long, 8 cm wide,
and 14.5 cm high. In order to minimize the influence of olfactory cues, the floor was carefully cleaned between each trial. Numerous distal cues (chairs, curtains, doors, posters, tables, shelves, lamps, and various small objects) were around the Y-maze in the testing room and were kept constant during the entire behavioural testing period. One hour before the beginning of the experiment, mice were brought to the experimental room under the same light and noise conditions as during the experimental phases. All experiments were conducted in the same room and in the same maze, with the same cues. A validated paradigm for different strains of mice (Dellu et al., 2000) was used and consisted of two phases as follows:

**Experiment I: Response to Novelty (2-min ITI)**

This experiment consisted of two trials separated by a 2-min intertrial interval. During the acquisition phase (trial 1), one arm of the Y-maze was closed with a guillotine door. The position of the closed arm was chosen randomly among the three arms. Each mouse was placed in one of the two other arms, facing the side opposite to the center of the maze, and was allowed to visit the two accessible arms of the maze for 5 min. At the end of the trial, the mouse was returned to its cage. During the retrieval phase (trial 2) animals had free access to all three arms for 5 min. The position of the novel arm was to the left of the starting arm (arm 1) for half of the animals and to the right for the other half (in a random order). The number and the duration of visits to new arm were recorded in each trial.

**Experiment 2: Memory (30 min and 1 hour ITIs).**

Following characterization of the response to novelty with a short, 2-min ITI, spatial memory was assessed using longer ITIs: 30 min and 1 h. The procedure followed during this phase was identical to “response to novelty” described above phase but the ITIs were longer.
RESULTS

Electrophysiological analysis.

We used 3 trains of HFS (100 Hz for 1s every 20 s) to achieve LTP in CA1 hippocampal slices after the basal period (Fig. 1). We then tested the effects of PTN on HFS-induced LTP in the hippocampal slices in the presence of PTN (3 μM) included in the perfusion solution for 10 minutes and 10 min prior to stimulation. We found that HFS efficiently induced LTP; however, we observed a significant different level of potentiation between the two groups (216.4±6.6% vs 187.2±8.2% in control and PTN respectively, P<0.01, t58=2.77). Moreover, potentiation was not maintained when PTN was previously present in the hippocampal slices. In slices treated with PTN, the EPSP Slope reached values near to baseline one hour after the HFS application (121.0±4.7%) whereas the potentiation in control samples was maintained (217.6±8.2%) (P<0.001, t58=-11.0) (Fig. 1). These data clearly establishes that PTN is a potent inhibitor of LTP in CA1 hippocampal slices.

Behavioral performance of PTN-/- mice.

To better understand the role of PTN in memory formation processes in vivo, we used the Y-maze test. We observed that both PTN-/- and WT (+/+ ) mice displayed novelty recognition when the ITI was 2 min indicating that both groups have normal behavior in the Y-maze task (data not shown). No differences between the two groups were observed in the recognition memory 30 minutes after prior exposure (training period) to the Y-maze (Fig. 2A). When we increased the time interval between the
training period and testing from 30 to 60 minutes, we observed that PTN-/- mice maintained the recognition memory observed at 30 min. However, WT (+/+) mice did not express preference for any of the arms of the Y-maze thus suggesting a complete disruption of 60 min-recognition memory (Fig. 2B).
DISCUSSION

In the present work, we demonstrate that application of a low concentration of PTN (3 μM) strikingly inhibits HFS-induced LTP in hippocampal slices from Sprague-Dawley rats, thus confirming the inhibitory role of PTN on LTP (Lauri et al., 1998). Moreover, we found that PTN -/- mice show significantly enhanced short-term memory when compared to WT (+/+) mice measured using the Y-maze test. Both results seem to indicate an important role of PTN in learning and memory processes. These studies are consistent with studies of Lauri et al. (1998) that showed, using methods and concentrations of PTN different from ours, that exogenous PTN injected into the CA1 layer of hippocampal slices inhibits tetanus-induced LTP. Moreover, other authors have demonstrated that mice overexpressing PTN in the hippocampal CA1 layer showed decreased LTP (Pavlov et al., 2002).

Nevertheless, contradictory results have been reported concerning PTN, LTP and learning and memory. Pavlov et al. (2002) previously described that PTN -/- mice, that showed enhanced LTP, displayed significant reduction in the water maze spatial memory task whereas mice overexpressing PTN learn faster than controls (WT, +/+ ) in the same task. These results apparently disagree with our findings. We observed that PTN -/- mice have significantly enhanced short-term memory compared to WT (+/+) mice using the Y-maze. One possible interpretation is that PTN -/- mice have improved short-term memory, however, when a consolidated memory is required for a task, such as in the water maze test, PTN -/- mice fail to develop long-term memory compared to WT mice.

The relationship between changes in LTP and learning and memory processes has been also a controversial topic in the literature. Many authors have demonstrated a correlation between memory improvement and enhancement in molecular processes.
related to LTP. For instance, NMDA antagonists or PKC and PKA inhibitors, which impair LTP, induce deficits in learning and spatial memory (Mathis et al., 1992; Bannerman et al., 1997; Sharifzadeh et al., 2005). Several studies have shown a clear correlation between enhanced LTP in mutant mice with improved learning (Tang et al., 1999; Malleret et al., 2001), but also with unaltered (Manabe et al., 2000) or impaired learning (Migaud et al., 1998). In this sense, it has been demonstrated that saturation of cellular mechanisms involved in LTP and spatial memory (Lynch, 2004) induced the impairment of LTP or spatial memory respectively (Lynch, 2004). For example, it has been demonstrated that HFS stimulation, which induces LTP, significantly increases the levels of expression of PTN in hippocampus (Lauri et al., 1996). It is noteworthy that in the present studies, the saturation phenomena has been taken into consideration and the *in vivo* and *in vitro* experiments were made in different conditions to avoid this problem.

Interestingly, RPTPβ/ζ deficient mice exhibit an age (maturation)-dependent impairment of spatial learning in the Morris water maze test and enhancement of LTP in the CA1 region in hippocampal slices, suggesting the PTN receptor, RPTPβ/ζ, plays an important role in memory function (Niisato et al., 2005). Our data is consistent with the data obtained in the RPTPβ/ζ deficient mice, since, PTN -/- mice having “unrestrained” the phosphatase activity of RPTPβ/ζ and, thus, decreased phosphorylation levels of RPTPβ/ζ substrates, exhibit enhanced learning in the Y-maze. This hypothesis is supported by recent evidence showing that the capacity of RPTPβ/ζ to dephosphorylate Y1105 on p190 RhoGAP is critical for memory formation (Tamura et al., 2006). This is judged to be very important since p190 RhoGAP is one of the RPTPβ/ζ substrates whose phosphorylation levels are significantly increased by PTN (Tamura et al., 2006).
In summary, our studies demonstrate that a low concentration of PTN inhibits tetanus-induced LTP in hippocampal slices. Our behavioural data suggest a possible role of PTN and the PTN/RPTPβ/ζ signaling pathway in hippocampus-dependent memory formation.

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REFERENCES


receptor enhances plastic changes in hippocampal synapses and modifies behavioral responses. Mol Cell Neurosci 15:534-546.


Tamura H, Fukada M, Fujikawa A, Noda M. 2006. Protein tyrosine phosphatase receptor type Z is involved in hippocampus-dependent memory formation.


Fig. 1. Effect of perfusion with PTN in LTP produced in rat hippocampal slices. The symbols represent fEPSP slope values in hippocampal slices from CA1 animals. After a basal 20 min period, slices were perfused with PTN (3 μM) for 10 minutes. Ten minutes after the perfusion of PTN, 3 trains of HFS (indicated by arrow) were applied in PTN perfused slices (filled circles, n=7) and control slices (open circles, n=5). Upper traces are samples of the fEPSPs recorded during the basal and final periods in both control (left) and PTN (right) groups. Calibration: 1 mV, 2 ms.
Fig. 2: Behavioural performance of WT (+/+) and PTN -/- mice in the Y-maze.

Figure show the percentage of the number of visits made to each arm by a time interval (ITI) of 30 minutes (A) and 60 minutes (B) after training period in WT (n=13, white bars) and PTN -/- (n=14, black bars). Comparison were made between both groups in visits to novel arm (* P < 0.05).