Prolactin Reduces Hippocampal Parvalbumin and GABA_A Receptor Expression in Female Mice

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Keywords

Hyperprolactinemia · Inhibition · β 2/3 subunit of GABA_A receptors · Parvalbumin-positive interneurons

Abstract

Introduction: Parvalbumin (PV)-positive cells are strategic elements of neuronal networks capable of influencing memory and learning processes. However, it is not known whether pituitary hormones may be related to PV expression in the hippocampus – a part of the limbic system with important functions in learning and memory. **Objective:** Since previous studies indicate that prolactin (PRL) plays a significant role in hippocampal-dependent learning and synaptic plasticity, we hypothesized that a rise in PRL levels can modify PV expression in the hippocampus. **Methods:** We employed biochemical, immunohistochemistry, and densitometry techniques – as well as a behavioural assay – in a hyperprolactinemia model using subcutaneous osmotic pumps in female mice. **Results:** PRL treatment via osmotic pump induced an increase in PRL receptor (PRL_R) expression in most regions of the hippocampus analysed by Western blotting and immunohistochemistry methods. Fluorescent densitometry analysis revealed that PV expression decreases in the same layers in the hippocampus following PRL treat-

ment, while double labelling immunostaining indicated close localization of PV and PRL_R in PV-positive interneurons. In addition, we found that PRL induced a reduction in the β 2/3 subunit of GABA_A receptor (GABA_AR) expression that was linearly correlated with the reduction in PV expression. This reduction in the β 2/3 subunit of GABA_AR expression was maintained in trained animals in which PRL treatment improved the learning of a spatial memory task. **Conclusions:** These data show, for the first time, that an increase in PRL level is associated with changes in key constituent elements of inhibitory circuits in the hippocampus and may be of relevance for the alterations in cognitive function reported in hyperprolactinemia.

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Published by S. Karger AG, Basel

Introduction

Prolactin (PRL) is a polypeptide hormone secreted from pituitary and extrapituitary tissue that has an important influence on the central nervous system [1]. Several experimental findings indicate that the hippocampus – a constituent structure of the limbic system with relevant functions in learning and memory [2] – may be influenced by PRL [3–5]. In line with this evi-

dence, an increase in PRL levels enhances hippocampal synaptic plasticity [6] and promotes changes in the circuit dynamics of the hippocampus, improving the performance of spatial learning [7]. Interestingly, patients with prolactinomas showed lower scores on memory tasks [8] and cognitive deficits in verbal memory and executive function [9], indicating that a pathological rise in serum PRL concentration has a deleterious effect on cognitive functions involving the hippocampus. Parvalbumin (PV) is a Ca^{2+} -binding protein that acts as a buffer to reduce intracellular Ca^{2+} levels [10]. PV-expressing interneurons are critical modulators of hippocampal activity [11]. They synchronize hippocampal network dynamics required for memory consolidation [12] and also play an important role in contextual fear learning [13]. However, the possible interactions between high PRL concentrations, PV-positive cells, and the GABAergic system in the hippocampus remain unknown. To address this issue, we used a mouse model of hyperprolactinemia by chronic PRL treatment via osmotic pump. Here, we report that PRL regulates hippocampal PV and GABA_A receptor (GABA_AR) expression and, as a result, has an impact on behaviour.

Materials and Methods

Animals

Experiments were carried out in adult female RjHan/NMRI mice (Janvier Labs, Le Genest Saint Isle, France) with institutional bioethics committee (Cajal Institute Ethics Committee on Human and Animal Experimentation and CSIC Ethics Committee) approval of the experimental protocols in accordance with Spanish (RD 53/2013) and European Union (63/2010/EU) legislation.

Experimental Groups

The experiments were performed in female (2–3 months of age) mice that were housed under a standard 12/12-h light/dark cycle, at 21–25°C and 40–60% humidity with access to food and water ad libitum. The nulliparous female mice were randomly divided into animals implanted with an osmotic pump filled with PRL (PRL) or vehicle (Vh).

Osmotic Pump Implant

Detailed methods of the procedures for the osmotic pump implant have been described previously [6]. In brief, under anaesthesia, osmotic pumps (Model 2001; Alzet, Cupertino, CA, USA) filled with sheep PRL (Sigma, St Louis, MO, USA) or Vh (0.9% NaCl) were implanted subcutaneously at 9:00 a.m. to release 150 µg PRL/day continuously (1 µL/h) for 7 days. All experiments were performed 7 days after the implantations.

Measurement of Prolactin

Full details of the procedures to obtain mice serum and PRL measurement by ELISA have been described previously [7]. In

brief, blood was collected from the submandibular vein and centrifuged, and serum was then taken and stored at –80°C. PRL concentrations were quantified using a prolactin mouse ELISA Kit (ab100736; Abcam), following the manufacturer's instructions.

Western Blot Analysis

Mouse brains were quickly removed, and both hippocampi were extracted in artificial cerebrospinal fluid at 4°C and gassed with a 95% O₂ and 5% CO₂ mixture at pH 7.3–7.4. Tissue lysis was performed in RIPA buffer: 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Igepal CA-630, 1 mM NaF, 0.1% Phosphatase Inhibitor Cocktail 2 (Sigma, #P5756), 0.1% Protease Inhibitor Cocktail (Sigma, #P8340), and 2 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 12,000 g for 15 min at 4°C, and the resulting supernatants were collected. Protein concentration was determined by using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein extracts (30–50 µg) were denatured in Laemmli's sample buffer containing SDS, β-mercaptoethanol, and bromophenol blue at 95°C for 8 min. The proteins were separated by 8–10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA, USA). The membranes were blocked by immersing in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The primary antibodies used were anti-GABA_A receptor (GABA_AR) β2/3 (1:1,000; #05-474) (Merck Millipore, Burlington), anti-PRL receptor (anti-PRL_R) (1:500; #MA1-610) (Thermo Fisher Scientific, Waltham), and anti-GAPDH (1:8,000; #AM4300) (Thermo Fisher Scientific, Waltham). The antibodies were incubated in blocking buffer overnight at 4°C. The membranes were washed with TBS-T and incubated for 1 h at room temperature with anti-mouse HRP (1:5,000; #115-035-003) (Jackson ImmunoResearch Laboratories, Baltimore, PA, USA). Chemiluminescence detection was performed with a detection kit (ECL SuperSignal West Dura; #34075) (Thermo Fisher Scientific, Waltham), and Western blot signals were obtained via LAS 3000 mini Imager (Fujifilm, Tokyo, Japan). Band density was quantified using ImageJ software (Version 1.50i, <https://imagej.nih.gov/ij/>) and expressed as a ratio of the protein of interest/GAPDH expression. Data were normalized to the vehicle group.

Tissue Processing and Immunohistochemistry

Animals were anesthetized by intraperitoneal injection of ketamine/xylazine 1:1 (0.1 mL/kg) and transcardially perfused with saline (25 mL) followed by 4% paraformaldehyde (100 mL), pH 7.4, in 0.1 M phosphate buffer. The brains were dissected and postfixed in paraformaldehyde for 4–8 h at 4°C, immersed in 30% sucrose for 48 h, and embedded in tissue-freezing medium (Neg-50; Thermo Fisher Scientific), by freezing in liquid nitrogen. Coronal cryosections were cut (20-µm thick) using a Microm HM 550 cryostat (Thermo Fisher Scientific, Waltham) and collected on SuperFrost Plus slides (Thermo Fisher Scientific, Waltham). After permeabilization with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 2 h at room temperature and blocking with 0.05% Triton X-100 in PBS and 5% normal goat serum (NGS) for 1 h at room temperature, the slides were then incubated with primary antibodies: mouse anti-GABA_AR β2/3 (1:250; #05-474) (Merck Millipore, Burlington), mouse anti-parvalbumin (1:1,000; #P3088) (Sigma, San Luis, USA), and rabbit anti-PRL_R (1:500; #170935) (Abcam, Cambridge,

UK). The antibodies were incubated in blocking buffer overnight at 4°C. All slides were incubated with either Alexa Fluor 488-labelled anti-mouse Ab (Thermo Fisher Scientific, #A11029) or Alexa Fluor 568-labelled anti-rabbit Ab (Thermo Fisher Scientific, #A11036). Nuclear staining was performed with DAPI (1:5,000; #D9542; Thermo Fisher Scientific, Waltham). The samples were extensively washed with PBS between steps. The sections processed in the absence of primary antibody showed a lack of noticeable non-specific staining in our experimental conditions (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000520279 for all online suppl. material). To support this negative control, we also analysed areas of the brain with very low PRL_R expression such as the caudate-putamen (online suppl. Fig. 1). The sections were analysed by confocal microscopy (LSM 800; Zeiss, Oberkochen, Germany) with a 40X oil immersion objective and quantified by ImageJ software (Version 1.50i; <https://imagej.nih.gov/ij/>), using the “integrated density” parameter. To perform the hippocampal quantifications, we selected 3 slides per animal (typically 8 mice for each condition) in a rostro-caudal extension approximately from interaural 1.98 mm and bregma –1.82 mm to interaural 1.62 mm and bregma –2.18, with a distance of 100 µm between slides (online suppl. Fig. 2, upper panel). We analysed the 3 main areas of both hippocampi – CA1, CA3, and dentate gyrus (DG) – distinguishing 2 layers per area, as follows: CA1 stratum pyramidale (SP) and stratum radiatum (SR), CA3 stratum pyramidale (SP) and stratum lacunosum (SL), and DG stratum moleculare (SM) and stratum granulosum (SG) (online suppl. Fig. 2, bottom panel). Three representative sections of each layer were measured in each hippocampus. Data were normalized to the vehicle group.

Barnes Maze

Three days after the osmotic pump implant, the mice were trained in the Barnes maze (BM, Fig. 6a). Detailed BM and training procedures have been described previously [7]. Prior to training, the animals were habituated to the testing room for 10 min. They were trained for 4 days, with 4 trials per day and an intertrial interval of 15 min. Between trials, the maze was wiped with 70% ethanol to avoid olfactory cues. Spatial memory retention was tested 24 h after the last training trial (probe, day 5; Fig. 6a). The number of holes checked before the mouse escaped into the target hole (Fig. 6a, b) and total distance travelled to locate the target hole (Fig. 6a, c) were used to assess the learning and motor performance.

Statistical Analysis

To perform statistical analysis (online suppl. Table 1), we first used the Shapiro-Wilk test to determine whether the distribution of the data was normal (parametric data) or not (non-parametric data). To establish differences between experimental conditions, we used Student's *t* (parametric) or Mann-Whitney U (non-parametric) tests to compare 2 experimental groups and univariate ANOVA (generalized linear models) to compare trained and untrained conditions. In order to analyse the relationship between PV cells and GABA_AR β2/3 expression in all of the different hippocampal regions, correlation analysis was performed using parametric (Pearson correlation; *r*) or non-parametric (Spearman Rho; *ρ*) correlation tests chosen based on the normality of the data. All data are expressed as mean ± standard error of the mean. For all analyses, values of *p* < 0.05 were considered statistically significant. Statistical analysis was performed using SPSS software.

Results

To investigate the influence of high serum levels of PRL on hippocampal PV-positive cells, we used a model with osmotic pump continuous infusion of PRL (150 µg/day for 7 days) or vehicle in nulliparous female mice. This treatment prompted a significant increase in serum levels of PRL compared with Vh (Fig. 1a), reaching values similar to those observed during lactation [14] or moderate pathological hyperprolactinemia [15, 16]. This rise in serum concentration of PRL was paralleled by a significant increase in long (PRL_R L) and short (PRL_R S) PRL_R isoform expression examined in the whole hippocampi by Western blot analysis (Fig. 1b). A more detailed analysis of PRL_R expression in different regions of the hippocampus by fluorescent microscopy using IHC (Fig. 1c) revealed that PRL treatment significantly increases PRL_R expression in all the regions studied. Relative fluorescence density of these immunosections was measured in SP and SR layers of CA1, SP and SL layers of CA3, and SG and SM layers of DG (Fig. 1d). These data indicate that chronic osmotic pump treatment with PRL induced the expression of PRL receptors in the main areas of the hippocampus except in the SM layer of DG.

We then studied PV expression in different areas of the hippocampus and its relationship with the increase in PRL. Double labelling immunostaining indicated close localization of PV and PRL_R in PV-positive cells (Fig. 2; online suppl. Fig. 3). In conjunction with this finding, confocal microscopy analysis revealed that PV expression decreased in most of the layers of the hippocampus in response to PRL treatment (Fig. 3a). We quantified this decrease by relative fluorescence density in CA1, SP; CA1, SR; CA3, SP; CA3, SL; DG, SG; and DG, SM (Fig. 3b). These data indicate that increasing PRL levels reduced PV expression in the main hippocampal areas following the same pattern as PRL_R distribution.

Previous findings indicate that the β subunit of GABA_AR is required for inhibitory transmission in the hippocampus, and β3 knockout impairs inhibitory input from PV but not somatostatin-expressing interneurons [17]. Consequently, we then determined the effect of hyperprolactinemia on the expression of this GABA_AR subunit. GABA_AR β2/3 expression throughout the hippocampus (Fig. 4a) and relative fluorescent density quantification indicates that GABA_AR β2/3 expression decreased in all quantified layers in CA1, SP; CA1, SR; CA3, SP; CA3, SL; DG, SG; and DG, SM (Fig. 4b). In line with the above results, we studied the correlation between PV and GABA_AR β2/3 subunit expression. Our analysis revealed a sta-

Fig. 1. Chronic PRL treatment increases PRL receptor expression in the hippocampus. **a** PRL levels were detected in serum by ELISA. Values represent mean $\pm$ SEM (Vh $n = 8$; PRL $n = 7$). Data were analysed by the Mann-Whitney U test (*** $p < 0.001$). **b** Western blot analysis of PRL_R expression in protein extracts from the hippocampus. Long (PRL_R L, 100 kDa) and short (PRL_R S, 40 kDa) isoforms of PRL receptors were measured, and representative immunoblots are shown. GAPDH was included for normalization. Measurements represent mean $\pm$ SEM (Vh $n = 8$, PRL $n = 8$). Data were analysed by Student's t test (* $p < 0.05$; ** $p < 0.01$). **c** Immu-

nofluorescent detection of PRL_R. Hippocampal sections were processed for PRL_R immunostaining and analysed by confocal microscopy. The arrows show PRL_R expression. Scale bar, 10 μ m. **d** Graphs showing relative fluorescence density analysis in different layers of CA1, CA3, and DG as indicated in the top panel. Values represent mean $\pm$ SEM (Vh $n = 7$; PRL $n = 6$). Data were analysed by Student's t test and Mann-Whitney U test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). PRL, prolactin; SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lacunosum; SG, stratum granulosum; SM, stratum moleculare.

tistically significant linear relationship between PV and GABA_AR $\beta 2/3$ expression in all the hippocampal regions studied (Fig. 5). These results suggest that an increase in hippocampal PRL levels has the capacity to influence key

constituent elements of inhibitory circuits in the hippocampus.

Prior experimental evidence indicates that (a) PV-expressing interneurons coordinate hippocampal network

Fig. 2. Parvalbumin-positive cells colocalize with prolactin receptors. Confocal laser-scanning micrographs of CA1 hippocampal sections from PRL-treated mice immunostained for PV-positive cells (Alexa 488, green), PRL_R (Alexa 568, red), and DAPI (blue). Scale bar, 20 μ m. Note the colocalization of the PRL_R on PV-positive structures (“merge” panel). The arrows indicate PV with PRL_R expression. PV, parvalbumin; PRL, prolactin.

dynamics required for memory consolidation [11, 12] and (b) PRL improves the learning of a spatial memory task in the acquisition stage [7]. Therefore, the next step was to study the correlation between inhibitory down-regulation by PRL and the performance of a hippocampus-dependent spatial memory task using the BM (Fig. 6a), which is a well-established test to evaluate spatial reference memory in mice [7, 18]. The PRL treatment with osmotic pump significantly decreased the number of errors before mice escaped into the target hole on day 2

(D2) of the acquisition phase (Fig. 6b) compared to Vh. This observation correlated with a decrease in the total distance travelled to reach the target hole (Fig. 6c) on the same day. In addition, we quantified hippocampal GABA_A β 2/3 expression by Western blotting in BM-trained versus untrained animals (Fig. 6d). In agreement with preceding results (Fig. 4), we found that PRL treatment reduced hippocampal GABA_A β 2/3 expression in both experimental conditions (Fig. 6d). Taken together, these findings suggest that an increase in the levels of serum

Fig. 3. Increase in PRL level decreases PV expression in the hippocampus. **a** Immunofluorescent detection of PV in hippocampal sections. Arrows indicate PV expression in different hippocampal areas. Scale bar, 10 μm. **b** Graphs showing relative fluorescence density analysis of CA1, CA3, and DG hippocampal areas (highlighted in the

top images at low magnification). Values represent mean ± SEM (Vh $n = 7$; PRL $n = 6$). Significant differences were established with Student's t test (** $p < 0.01$; *** $p < 0.001$). PV, parvalbumin; PRL, prolactin; SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lacunosum; SG, stratum granulosum; SM, stratum moleculare.

PRL enhanced the performance of a spatial memory task and induced a persistent reduction in the hippocampal inhibitory network activity resulting in an improvement in the learning process.

Discussion

PRL is a hormone with a broad spectrum of action and is involved in many physiological processes throughout the mammalian organism. In addition to the classical function of PRL in galactopoiesis, lactogenesis, and reproductive behaviour, cumulative clinical and experimental evidence indicates that PRL modulates diverse brain processes including hippocampal-dependent learning and memory [3, 5, 7, 19–21]. Furthermore, hyperprolactinemia has been related to cognitive impair-

ments [8, 9] associated with structural changes in the hippocampus [9]. The present results show that chronic PRL infusion with minipumps significantly increases both long and short PRL_R isoform expression in most hippocampal regions, suggesting that this area of the brain is highly susceptible to the activity of this hormone. Beyond the variations of serum PRL levels between species and sex, different physiological and pathological conditions can lead to a state of hyperprolactinemia. Late pregnancy and lactation together represent the most common cause of physiological hyperprolactinemia [22–24]. In mice, PRL levels remain high during lactation, with peaks in response to suckling [24], and then progressively decrease [14]. Under our experimental conditions, the mean concentration of serum PRL was very close to that observed by other authors during lactation in mice [14]. Another point to consider is that patholog-

Fig. 4. Increase in PRL levels reduces GABA_A β2/3 expression in different areas of the hippocampus. **a** Immunofluorescent detection of GABA_A β2/3 in hippocampal sections of vehicle (Vh)- or PRL-treated mice. The arrows indicate GABA_A β2/3 expression. Scale bar, 10 μm. **b** Graphs showing relative fluorescence density analysis of CA1, CA3, and DG hippocampal areas (highlighted in

the top images at low magnification) in both experimental conditions. Values represent mean ± SEM (Vh *n* = 7; PRL *n* = 6). Significant differences were established by Student's *t* test and Mann-Whitney U test (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). PRL, prolactin; SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lacunosum; SG, stratum granulosum; SM, stratum moleculare.

ical hyperprolactinemia involves a wide range of PRL concentrations depending on the aetiology in question (e.g., pituitary adenoma, drugs, renal failure, and liver cirrhosis) [25]. Through the treatment with osmotic pumps, we doubled normal serum PRL levels, obtaining a moderate degree of hyperprolactinemia [15, 16]. We conclude that the present experimental model is useful to study the isolated effect of PRL in the hippocampus or other brain areas in physiological conditions (e.g., lactation) and in the initial stages of different pathological processes.

Previous data indicate that PRL influences learning of a spatial memory task modifying the oscillatory activity of the hippocampus [7]. In addition, PV-expressing interneurons are involved in the synchronization of the hippocampal network [11, 12], and they contribute to the

generation of network oscillations [26, 27] as well as mediating neocortical-hippocampal interactions that are necessary for memory consolidation [28]. We present new evidence indicating that the PRL_R colocalizes with PV-positive cells, and an increase in PRL concentration induces a reduction in PV expression in the main hippocampal areas. Therefore, it is feasible that a change in hippocampal rhythmic activity during learning may be regulated via direct action of PRL on PV-positive interneurons. Since successful memory consolidation requires coordinated hippocampal-neocortical communication mediated by PV-expressing interneurons [28], the present data reveal the PV-positive cell as a target of PRL that was not previously known. This finding may explain why changes in PRL levels can improve hippocampal-dependent learning and memory.

Fig. 5. Hippocampal PV-positive cells and GABA_A β 2/3 expression correlation after chronic PRL administration. A linear relationship can be seen between PV-positive cells and GABA_A β 2/3 expression in different CA1, CA3, and DG hippocampal areas. As shown, there was a significant positive correlation between the expression of PV-positive cells and GABA_A β 2/3 in all the hippocampal regions studied (CA1 SP: $\rho = 0.764$, $**p < 0.01$, $n = 13$; CA1 SR: $r = 0.781$, $**p < 0.01$, $n = 13$; CA3 SP: $r = 0.786$, $**p < 0.01$, $n = 13$; CA3 SL: $r = 0.578$, $*p < 0.05$, $n = 13$; DG SG: $\rho = 0.731$, $**p < 0.01$, $n = 13$; DG SM: $r = 0.806$, $**p < 0.01$, $n = 13$). PV, parvalbumin; PRL, prolactin; SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lacunosum; SG, stratum granulosum; SM, stratum moleculare.

GABA_ARs are responsible for fast inhibitory neurotransmission in the mammalian brain [29] and comprise different subunit families and isoforms [30]. This diversity gives rise to a vast heterogeneity in the composition of GABA_A subtypes and confers upon GABA_AR with particular functional and pharmacological properties [31, 32]. In the hippocampus, the β 3 subunit is essential for GABA_AR to function adequately [17]. Interestingly, the lack of β 3 is enough to impair inhibitory transmission, preferentially affecting PV-positive interneurons rather than somatostatin-positive ones, highlighting the critical role of the β 3 subunit of GABA_AR in hippocampal inhibitory transmission [17]. Our findings show that an increase in PRL concentration reduces β 2/3 subunit expression of GABA_AR in different regions of the hippocampus that was paralleled by a linear correlation with a reduction in PV expression in the same regions. In addition, the

present results show that an increase in serum PRL levels enhances the performance of a spatial memory task in agreement with previous results [7]. Notably, no significant changes were observed in the PRL reduction of β 2/3 subunit expression of GABA_AR in the whole hippocampus in trained versus untrained animals. Since the lack of the β 3 subunit preferentially affects PV-positive cells [17], it is feasible that PRL affects hippocampal inhibitory transmission, modifying the excitatory-inhibitory balance necessary to maintain learning-induced synaptic modifications involved in long-term memory induction and maintenance [33].

In mammals, the hippocampus plays a key role in acquisition, storage, and retrieval of spatial information [2, 34], and this process results in an extensive remodelling of neural networks [35]. For this, interactions between GABAergic interneurons and excitatory pyramidal neu-

Fig. 6. Prolactin treatment improves spatial learning in the Barnes maze and reduces GABA_A β2/3 expression in untrained and trained animals. **a** Schematic representation of error count prior to entry into the escape box in the Barnes maze (left) and schedule of the training (right). The treatment began 3 days before training day 1 and lasted until day 5 (probe). **b** Graph showing the decreasing number of errors quantified across days of training in mice treated with saline (Vh) or PRL. Significant differences on day 2 (D2) were found between the 2 treatments (* $p < 0.05$, PRL $n = 9$, Vh $n = 9$). **c** Comparison of the average daily distances for mice

treated with saline (Vh, $n = 9$) or PRL (PRL, $n = 9$). There were significant changes in this parameter during the learning process (* $p < 0.05$, PRL $n = 9$, Vh $n = 9$). **d** Western blot analysis of GABA_A β2/3 (55 kDa β2 and 57 kDa β3) levels in the hippocampus in trained and untrained animals. Representative immunoblots are shown. Significant differences were established by Student's t test and ANOVA. Values represent mean $\pm$ SEM (untrained: *** $p < 0.01$, Vh $n = 8$; PRL $n = 8$; trained: *** $p < 0.01$, Vh $n = 7$; PRL $n = 7$; ANOVA: $p > 0.05$). PRL, prolactin.

rons are necessary [11, 36–39] in order to operate at synaptic and circuitual levels in the hippocampus. In this regard, long-term potentiation (LTP) is a well-established model of the synaptic mechanism underlying some forms of learning and memory [40, 41], and recent data indicate that PRL improves LTP through mechanisms that include the modulation of inhibitory activity mediated by GABA_AR in order to modify the synaptic strength [6]. Interestingly, lactation modifies GABA neuronal activity and increases the density of GABAergic synapses in extra-hippocampal brain areas [42, 43]. Furthermore, changes in GABA_AR expression in the hippocampus have been observed during pregnancy and the postpartum period [44]. It is reasonable to assume that PRL can modulate hippocampal-dependent learning and memory in physiological conditions, given that (a) interneurons are strategically placed in the hippocampal network in order to synchronize its activity [11, 12]; (b) inhibitory synaptic plasticity and the GABAergic system are essential for learning and memory formation [45] and can be affected by PRL ([6]; present results); and (c) an increase in PRL levels is associated with changes in key constituent elements of hippocampal inhibitory circuits (present results).

The statement in the previous paragraph may seem to contradict the clinical evidence from patients with hyperprolactinemia induced by prolactinoma that have shown a lower score on memory tasks [8] and cognitive deficits in verbal memory and executive function [9]. However, it should be noted that in both experimental hyperprolactinemia (as in the present study) and physiological hyperprolactinemia, elevated PRL levels are maintained for a limited time. Therefore, it is possible that under conditions of pathological hyperprolactinemia, a chronic decrease in inhibitory activity may have deleterious effects on cognition. Future studies, beyond the scope of the present work, will be necessary to elucidate the cellular and molecular mechanisms underlying the interaction between PRL and the GABAergic system, as well as their impact on hippocampal network activity, in more complex pathological condition such as prolactinoma.

PRL is a pleiotropic hormone responsible for important changes in several brain processes in which the underlying mechanisms of action are not fully understood. The present study highlights the relevant role of PRL in regulating inhibitory transmission by modifying the expression of PV and $\beta 2/3$ subunit of GABA_AR and identifies PRL as an important neurohormonal regulator of GABAergic function at the molecular and behavioural level. These findings will provide new insight into the role

of PRL and the way in which hippocampal GABAergic function may be modified during physiological and pathological hyperprolactinemia.

Acknowledgment

We would like to thank Nick Guthrie for his editorial services, reviewing the text prior to submission.

Statement of Ethics

Studies involving animals have been approved (Reference No. PROEX 036/18) by the following institutional animal care and use committees: (1) the Cajal Institute Ethics Committee on Human and Animal Experimentation, (2) the Spanish National Research Council (CSIC) Ethics Committee, and (3) the Council for the Environment, Regional Planning, and Sustainability, Madrid, Spain. The studies follow internationally recognized guidelines in accordance with Spanish (RD 53/2013) and European Union (63/2010/EU) legislation.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by grants BFU2017-88393-P and PID2020-116327GB-I00 funded by MCIN/AEI/10.13039/501100011033, Spain and ERDF “A way of making Europe”, EU, to E.D.M.

Author Contributions

S.M., B.M.-R., S.E., and M.F. acquired, analysed, and interpreted data and revised the manuscript critically for intellectual content. E.D.M. conceptualized and designed the study, interpreted the data, wrote the original draft, and revised the manuscript. All authors approved the final version for publication and agreed to be accountable for all aspects of the work.

Data Availability Statement

All data generated or analysed during this study are included in this article or its online supplementary material files. Further enquiries can be directed to the corresponding author.

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