Prolactin administration during early postnatal life decreases hippocampal and olfactory bulb neurogenesis and results in depressive-like behavior in adulthood

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A R T I C L E   I N F O

Article history:
Received 3 August 2013
Revised 4 October 2013
Accepted 8 October 2013
Available online 18 October 2013

Keywords:
Neuroendocrine
Neonate
Anxiety
Stress
Development
BrdU

A B S T R A C T

Tight regulation of hormone and neurochemical milieu during developmental periods is critical for adequate physiological functions. For instance, activation of peptide systems during early life stress induces morphological changes in the brain resulting in depression and anxiety disorders. Prolactin (PRL) exerts different actions within the brain; it regulates neurogenesis and modulates neuroendocrine functions in the adult. However, PRL effects during early postnatal life are hardly known. Therefore, we examined whether neonatal administration of PRL influences cell survival in the hippocampal dentate gyrus (DG) and in the olfactory bulb (OB) and whether such influence results in behavioral consequences in adulthood. PRL-treated rat pups (13 mg/kg; PND1 to PND14), injected with BrdU at postnatal day 5 (PND5), showed a decrease in the density of DG BrdU/DCX and BrdU/NeuN-positive cells that survive at PND15. Similarly, PRL treatment decreased the density of BrdU+ cells in the OB compared with VEH. Fluorojade B analysis showed no significant changes in the amount of cell death in the DG between the groups. Postnatal PRL administration induced a passive coping strategy in the forced swimming test in male and female adult rats when compared with control and vehicle groups. Corticosterone endogenous levels at PND12 were not affected by PRL or VEH treatment. Altogether, these results suggest that opposed to its effects in the adult, postnatal PRL treatment affects neurogenesis and results in psychopathology later in life. High PRL levels, as observed in neonates under several pathological states, might contribute to detrimental effects on the developing brain.

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Introduction

Abnormal activation of hormone and neurochemical systems including the hypothalamic–pituitary–adrenocortical (HPA) axis during critical developmental periods results in a high vulnerability to a range of behavioral and physiological disorders over a lifetime. For instance, the secretion of multiple hormones such as corticotrophin releasing hormone (CRH), glucocorticoids (GC), oxytocin (OXT) (Joels and Baram, 2009) and prolactin (PRL) (Seggie and Brown, 1975) due to early stress exposure induces several alterations in the brain, increasing the possibility to suffer anxiety and depressive disorders in adulthood (Chapman et al., 2004; Cohen et al., 2006; Heim et al., 1997). During the first postnatal weeks a stress hyporesponsive period takes place in the rodents, providing pups with low corticoid and neuropeptide levels, a requisite for optimal neuronal development (Sapolsky and Meaney, 1986; Schapiro, 1962). Alteration of GC and other hormonal factors such as CRH has been involved in the manifestation of the psychophysiological effects observed after adverse events (van Oers et al., 1998). However nothing is known about the effects of increased concentrations of PRL during this early time window.

PRL regulates a large number of physiological processes, from immunity to stress (Bole-Fysot et al., 1998). In the adult, PRL regulates HPA axis activity and reduces anxiety- (Torner et al., 2001) and depressive-like behaviors (Drago et al., 1990). Different stressors induce PRL release from the pituitary (Seggie and Brown, 1975), and circulating PRL enters the CNS through the choroid plexus (Walsh et al., 1987). This hormone is present in fetal and maternal serum (Bole-Fysot et al., 1998) and in maternal milk, from where it passes to the neonate into its circulation (Grosvener and Whitworth, 1983). PRL is a survival factor during embryogenesis (Nguyen and Zhu, 2009), and PRL receptor density is very high in fetal tissues including the brain but decreases in adulthood (Royster et al., 1995). Thus, PRL could be a candidate to influence some aspects of brain development.
The hippocampus is one of several limbic brain structures implicated in the pathophysiology of depressive and anxiety disorders (Campbell and Macqueen, 2004). Many studies demonstrate that stress and depression lead to reductions of the total volume of this structure and structural plasticity (Fuchs et al., 2004). Maintaining normal hippocampal neurogenesis is a homeostatic mechanism involved in the pathogenesis of adverse early life programming, depression and anxiety disorders (Korosi et al., 2011; Mirescu et al., 2004). Both classical antidepressants and selective serotonin re-uptake inhibitors promote hippocampal neurogenesis (Malberg et al., 2000). Moreover, decreased hippocampal neurogenesis during development is related to depressive-like behaviors in adulthood (Brummette et al., 2006; Lajud et al., 2012). PRL counteracts the stress-induced decrease of hippocampal neurogenesis (Torner et al., 2009) and stimulates adult neurogenesis in the SVZ (Larsen and Grattan, 2010). Despite the extensive characterization of PRL functions in the adult brain, few studies have evaluated its effects during the perinatal period. Here, we studied the influence of PRL treatment during early postnatal life on hippocampal neurogenesis and the long-lasting behavioral effects of this treatment in the adult.

Materials and methods

Animals

Sprague-Dawley dams were housed in standard temperature controlled rooms with a 12-hr light/dark cycle (light on at 0700 h) and free access to food and water. The day of delivery was designated postnatal day zero (PND0). At PND1 litters were adjusted to eight pups, four males and four females, with as little disturbance to mothers and pups as possible, this, in order to eliminate confounding effects of litter size or differences in maternal care due to sex or competition (i.e. licking and grooming). From PND1 to PND14, litters received either subcutaneous prolactin treatment (Ovine PRL, Sigma-Aldrich, St Louis, MO, U.S.A., 13 mg/kg in 0.01% NaOH, 0.9% Saline) or vehicle treatment (VEH, 0.01% NaOH, 0.9% Saline), or were left undisturbed (AFR/CONT). PRL dose was adjusted every five days based on average pup weight; no signs of irritation were observed around the injection site. All litters were animal-facility reared and underwent routine cage cleaning once a week. Some of the animals were sacrificed at PND12 for hormone analysis or at PND15 for neurogenesis evaluation; the rest of the animals were weaned at PND21 and group-housed according to sex and treatment (3–5 per cage). Rats remained undisturbed until adulthood. Each group/experiment included animals from at least three different dams, in order to decrease litter and maternal care effect. All experiments were approved by the ethics committee and performed in accordance with the official regulations for use and care of laboratory animals of Mexico (NOM-062-ZOO-1999) and the guidelines of the US National Institutes of Health. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Neurogenesis evaluation

Pups were injected twice, 8 h apart, with intraperitoneal (i.p) bromodeoxyuridine (BrdU, Sigma-Aldrich, St Louis, MO, U.S.A., 50 mg/kg) at PND5. At PND15 (CONT males n = 4, CONT females n = 6, VEH males n = 6, VEH females n = 4; PRL males n = 5, PRL females n = 4), pups were euthanized with pentobarbital anesthesia and were transcardially perfused. Their brains were removed, post-fixed, and stored at −30 °C until processing. Body weight and size were measured and adrenals were dissected and weighted for stress parameter evaluation.

Immunostaining

To visualize BrdU-positive cells, sagittal sections were cut in 40 μm systematic-random series and immunostained as previously described (Lajud et al., 2012; Torner et al., 2009). All immunostaining and immunofluorescence procedures were performed on free floating sections and all antibody incubations were carried out in a PBT solution (phosphate buffer + 4% Triton X-100, Sigma-Aldrich, St Louis, MO, U.S.A.) containing 5% horse serum (GIBCO, Paisley, Scotland). After permeabilization (10 min, PB + 10% H2O2 + 4% Triton X-100), sections were rinsed three times (PB × 3 × 10 min), incubated for 2 h in formamide (50% in SSC, Sigma-Aldrich, St Louis, MO, U.S.A.) at 74 °C and washed for 5 min in pre-heated SSC buffer. DNA denaturation was performed in HCL (1 N) for 30 min at 37 °C. Sections were incubated for 10 min in Borate Buffer (pH 8.4) and rinsed three times in PB (3 × 10 min). For BrdU peroxidase immunostaining we used a mouse anti-BrdU IgG (1:5000, Roche Applied Sciences, Indianapolis, IN, U.S.A.) for 24 h at 4 °C. Brains were rinsed in PB (3 × 10 min), incubated for 2 h in a biotinylated secondary goat anti-mouse IgG (1:750, Vector laboratories, Burlingame, CA, U.S.A.), washed (PB × 3 × 10 min) and further incubated with the avidin–biotin peroxidase complex solution (5 μl/ml in PBT, Vectastain ABC kit, Vector laboratories, Burlingame, CA, U.S.A.) for 90 min. Immunostaining was visualized using DAB staining kit for 10 min (Vector laboratories, Burlingame, CA, U.S.A.). Sections were mounted on gelatin coated slides and coverslipped. No dehydration processes were needed. For triple immunofluorescence, we used a primary rat anti-BrdU IgG (1:200, ABD Serotec, Oxford, UK) O.N. at room temperature. Sections were washed (5 × 10 PB), incubated for 3 h in a secondary donkey anti-Rat IgG (1:200, Jackson Immuno research, Pennsylvania, PI, U.S.A.) and rinsed tree times in PB (10 min). Sections were then incubated O.N. in a mixture of mouse anti-NeuN IgG (1:300, Millipore, Temecula, CA, U.S.A.) and rabbit anti-DCX (1:300, Millipore, Temecula, CA, U.S.A.), washed (3 × 10), incubated for 2 h in a mixture of a fluorescent secondary antibody (1:500, Alexa 647 goat anti-mouse IgG Jackson Immuno research, Pennsylvania, U.S.A.) and a biotinylated donkey anti-rabbit IgG (1:300, Vector laboratories, Burlingame, CA, U.S.A.). Sections were then rinsed tree times in PB (3 × 10 min) and incubated for 90 min with the fluorescent avidin–FITC substrate kit (Vector laboratories, Burlingame, CA, U.S.A.), washed (3 × 10 min in PB), mounted and coverslipped with DAKO mounting media (DAKO, Glostrup, Denmark). Negative controls without primary antibodies were carried out for all Immunostaining and immunofluorescence protocols. Negative controls for mouse and rat IgG cross reactivity were also performed to determine working dilutions.

BrdU-labeled cell counting

For hippocampal BrdU + nuclei number estimations in CONT, VEH and PRL-treated animals, sections were evaluated as described (Verina et al., 2007; Lajud et al., 2012). The series of brain sections (10–12 slices, 240μm apart one another) was used for generating stereological counts of BrdU-positive nuclei along the extent of the hippocampus. The boundaries of the granular (GL) and subgranular layers (SGL) of the DG were digitally outlined with the aid of image analysis software (Axiovision 4.6, Karl Zeiss). Only BrdU-positive nuclei within the outlined areas were considered in our counts. Total BrdU+ nuclei numbers were obtained from the dorsal and ventral blades of the DG. GL volume was done according to the Cavalieri principle, where the volume is the sum of the areas of each section of thickness t (40 μm). Total cell density (BrdU+ nuclei/mm³) was estimated from these parameters. To evaluate treatment effects on the subventricular zone and rostral migratory pathway, the density of BrdU + nuclei (BrdU+ nuclei/mm³) was analyzed from three optical frames of 4–5 sections in the granular layer of olfactory bulb of each animal, and these results were averaged. Because BrdU-labeled cell counting was
performed in an age prior to the onset of puberty, the above data were considered from both sexes. A separate set of animals was used to confirm that the number of BrdU+ nuclei was similar in both sexes (Males: $2717.1 \pm 312.3 \text{ BrdU+ nuclei/mm}^3$, n = 8 and Females: $2554.5 \pm 228.8 \text{ BrdU+ nuclei/mm}^3$, n = 10; p = 0.68).

Developmental cell death evaluation

Fluorojade B (FJ) staining was performed as previously described (Tejadilla et al., 2010). Slides were dehydrated, fixed with a potassium permanganate solution ($\text{KMnO}_4$ 0.1%), incubated in a FJ solution ($2 \mu \text{g/ml; Millipore, Temecula, CA}$) for 1 h at 32 °C, dried for 20 min at 72 °C, mounted and coverslipped with Cytoseal. Dorsal and ventral blades of the DG were photographed, and the mean relative optical density in the green channel (OD) was determined with the aid of image analysis software (Axiovision Rel 4.0, Karl Zeiss). Since developmental cell death decreases along lifespan, a different group of animal facility reared (two months old) control males was included for comparative purposes (n = 8).

Hormone evaluation

For assessment of corticosterone (CORT) and PRL, PND12 CONT pups were separated from the dam, rapidly decapitated, and plasma samples were obtained. Handling and sacrifice procedures were kept to a minimum time (30–60 sec) for each pup. VEH- and PRL-treated pups were injected and left to recover for 1 h before sacrifice. CORT levels were assessed in duplicate with a commercially available ELISA kit (Assay Designs, Ann Arbor, MI). Plasma prolactin was assayed by radioimmunoassay (RIA) using a selective antibody (5-8) purchased from A. F. Parlow (National Hormone and Pituitary Program [NHPP; Torrance, CA, U.S.A.]), and the respective radioactive tracer ($^{125}\text{I-}r\text{PRL}$) from NEN-Dupont (Boston, MA, U.S.A., specific activity $39 \mu \text{Ci/} \mu \text{g}$) according to standard procedures. Briefly, plasma samples ($20 \mu \text{l}$) were added to RIA phosphate saline albumin buffer ($0.01 \text{ M sodium phosphate, 0.14M NaCl, 0.1% bovine serum albumin}$), and final volume of samples and rPRL standards was $50 \mu \text{l}$. Final dilution of the anti-rPRL used was 1:15,000 ($100 \mu \text{l/tube}$) and the radioactivity added was around $2000 \text{ cpm/50 } \mu \text{l/tube}$. Samples were incubated overnight at room temperature and a second antibody ($1/40$ dilution, anti-rabbit IgG; Sigma-Aldrich Chemie) was added ($100 \mu \text{l/tube}$) and tubes were

Fig. 1. Prolactin administration decreases hippocampal BrdU labeled nuclei that survive at PND15. VEH or PRL was administered daily (from PND1 to PND14) to selected litters while other litters were left undisturbed (CONT). BrdU was injected to all the pups at PND5, and they were allowed to develop for 10 days. Left panels show representative images of BrdU immunostaining of the DG of CONT (A), VEH- (B) and PRL-treated (C) neonates (scale bar: 100 \( \mu \text{m} \)). Insets show higher magnifications of arrow pointed cells. The right panels show the stereological estimations of BrdU+ nuclei density (number of nuclei/volume) $\text{MEAN} \pm \text{SEM}$. ANOVA, $^*p < 0.01$ vs CONT, $^{**}p < 0.01$ vs VEH (DENSITY: ANOVA $\eta^2 = 0.48$, CONT vs PRL $d = 2.52$). VEH: Vehicle, n = 10; PRL: Prolactin, n = 9; CONT: Control, n = 10; GL: granular zone; SGL: subgranular zone.
incubated for 30 min. The samples were centrifuged (4°C, 30 min) after adding 6% polyethylene glycol (Sigma-Aldrich, St Louis, MO, U.S.A.). Supernatants were carefully removed and the radioactivity remaining in the precipitates was quantified using a gamma counter. All samples were run in one assay; intra-assay variation was 6.73% and limit of sensitivity was 0.53 ng/ml.

Behavioral tests

Adult rats were subjected to the elevated plus maze (EPM) as previously described (Lajud et al., 2012). The light intensity in the open and closed arms was set to 65 and 6 lx, respectively, with the neutral zone set at 50 lx. Animals were tested between 0900 h and 1200 h. Their behavior was recorded during a 5-min exposure period. Percentage of entries into the open arms and percentage of time spent on the open arms were evaluated.

For the forced swimming test (FST), rats were placed in water (21°C) in a plastic cylinder (50 cm tall × 30 cm diameter) for 10 min. Time spent struggling, swimming or floating was scored, according to the parameters previously described (Ebner et al., 2005).

Statistical analysis

Data are presented as group means ± SEM. Statistical analyses were performed using GB-stat V6.0 software (Dynamic Microsystems, Silver Spring, MD, U.S.A.). All data were analyzed using a one-way or two-way ANOVA and the Newman–Keuls post hoc test was used to determine group differences. Statistical significance was set at p ≤ 0.05. To estimate effect size, data were analyzed with eta squared for ANOVA and Cohen’s d for pair wise comparisons.

Results

PRL administration decreases hippocampal neurogenesis in the neonate

PRL treated pups showed no significant differences but a trend to decrease (F2,26: 2.78, p = 0.07, \(\eta^2 = 0.17\)) the estimated total number of BrdU+ nuclei in the hippocampal SGZ (CONT: 10,780 ± 696, VEH 11,342 ± 1265 and PRL 8414 ± 555 BrdU+ nuclei). Conversely, it caused a 27.8 ± 7.9% increase in the DG volume (F2,26: 4.66, \(p = 0.01\), \(\eta^2 = 0.26\); CONT 4.2 ± 0.1, VEH 4.1 ± 0.4 and PRL 5.4 ± 0.3 mm³) and a significant decrease (44.5 ± 7.2%) in BrdU+ nuclei density (F2,26: 12.34, \(p = 0.0002\), \(\eta^2 = 0.48\)) compared with CONT and VEH groups (Fig. 1). Because BrdU-labeled cell counting was performed in an age prior to the onset of puberty, the above data were considered from both sexes, however sexual dimorphism was evaluated. Two factor ANOVA showed a significant effect of treatment (F2,26: 11.82, \(p = 0.0003\), \(\eta^2 = 0.47\)) but no effect of sex (F1,26: 0.51, \(p = 0.47\), \(\eta^2 = 0.01\)) or interaction (F2,26: 1.12, \(p = 0.34\), \(\eta^2 = 0.04\))

Ten days after BrdU injection (PND15), newly generated cells surviving in the hippocampus of CONT, VEH, or PRL-treated neonates were detected primarily in the SGL of the dentate gyrus. Double immunostaining revealed that many BrdU+ nuclei co-localize with the mature neuronal marker NeuN (Fig. 2) and fewer co-localize with DCX, although at this stage immature neurons can express both DCX and NeuN markers. No differences were observed in the BrdU+/DCX+ and BrdU+/NeuN+ proportion between treatments.

Fig. 2. Cellular phenotype of BrdU labeled nuclei that survive at PND15. VEH or PRL was administered daily (from PND1 to PND14) to selected litters while other litters were left undisturbed (CONT). BrdU was injected to all the pups at PND5, and they were allowed to develop for 10 days (A). Upper left panels show single confocal planes of BrdU+, DCX+ or NeuN+ cells in the DG in a section colabeled for NeuN, DCX and for BrdU ( insets on the right), in CONT and PRL pups. (B). Upper right panels show representative images of the co-localization ( merge) of BrdU+ ( red) and NeuN+ ( blue) and of BrdU+ ( red) and DCX+ ( green) in the DG of CONT and PRL-treated pups ( insets on the right). Merged images include orthogonal views of x–z and y–z planes to confirm colocalization (C). Representative confocal photomicrograph illustrating the spatial distribution of BrdU+ nuclei ( red), DCX+ cells ( green) and NeuN+ neurons ( blue) and their co-localization ( merge; BrdU+/DCX+ in yellow; BrdU+/NeuN+ in magenta) in the hippocampal DG of CONT and PRL neonates at PND15. Merged images include orthogonal views of x–z and y–z planes to confirm colocalization (scale bar: 50 μm; inset, magnification of a selected region). GL: granular zone; SGL: subgranular zone.
**PRL administration effects on DG developmental cell death**

Because the decrease of granule cell survival in PRL-treated pups could be due to an increase in cell death, we analyzed the amount of optical density (OD, Green fluorescence) produced by Fj-B staining (Fig. 3). CONT and VEH, but not PRL, neonates showed increased staining in the SGZ compared to PND60 adult male controls (F3,28: 4.88, p = 0.007, η² = 0.34) derived from developmental cell death. Analysis excluding the adults showed non-significant differences found between CONT, VEH or PRL-treated neonates (F2,21: 3.43, p = 0.051; η² = 0.24; no post-hoc differences). Thus, our results indicate that PRL treatment does not induce an increase in developmental cell death in the DG.

**Effects of PRL administration on olfactory bulb cell survival**

PRL increases the number of granular cells from the olfactory bulb (OB) of the neonates at PND15 (Fig. 4). VEH treatment induced a significant increase in the density of BrdU+ nuclei in the OB compared with the CONT group, most likely due to handling effects. In contrast, PRL treatment induced mild increases in the estimated density of BrdU+ nuclei compared with CONT and significant decreases compared with VEH treatment (F2,21: 27.9, p = 0.0001; η² = 0.72, Fig. 4). This suggests that PRL is counteracting the increase of granular cell density in the OB induced by handling procedures.

**PRL administration during early life results in a passive coping strategy in both male and female adults; and induces a mild anxiogenic effect only in males**

Adult males postnatally treated with PRL showed a tendency to increase anxiety-like behavior in the EPM compared with control and VEH-treated groups because PRL-treated males showed a lower percentage of entries (Table 1) to the open arm (F2,21: 3.46, p = 0.050, η² = 0.24). In adult females, postnatal PRL treatment did not modify anxiety-like behavior (Table 1). However, a passive coping strategy on the FST was observed in both male and female adults (Fig. 5). PRL-treated male rats showed decreased time struggling (F2,21: 6.18, p = 0.007, η² = 0.37) and increased time floating (F2,21: 3.87, p = 0.03, η² = 0.27) compared with control and VEH-treated males (Fig. 5).

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*Fig. 3.* Prolactin has no effect on hippocampal developmental cell death. VEH or PRL was injected daily (from PND1 to PND14) to pups, and another group was left undisturbed (CONT). Developmental cell death was evaluated at PND15. Left panel shows representative confocal photomicrograph of a Fj-B staining in the hippocampal DG (scale bar: 100 μm) (A). Right panels show higher magnifications of CONT, VEH and PRL-treated neonates. Lower panels show OD quantification of Fj-B in the DG of all the groups (B), and normalized data as percent of decrease versus VEH group (C). MEAN ± SEM, ANOVA *p ≤ 0.05 vs CONT; *p ≤ 0.05 vs VEH (DENSITY: ANOVA η² = 0.24, CONT vs PRL d = .83, VEH vs PRL d = 1.50). VEH: Vehicle, n = 8; PRL: Prolactin, n = 8; CONT: Control, n = 8; GL: granular zone; SGL: subgranular zone.
Similarly, PRL-treated females displayed a shorter struggling time ($F_{2,21}: 4.32, p = 0.02, \eta^2 = 0.29$) and increased the time spent floating ($F_{2,21}: 8.70, p = 0.001, \eta^2 = 0.45$), compared with control and VEH counterparts (Fig. 5). Additional analysis was conducted in order to evaluate sex effect as an independent factor. In the forced swimming test we observed a significant effect of sex on struggling (Treatment: $F_{2,42}: 9.63, p = 0.0004, \eta^2 = 0.26$; Sex: $F_{1,42}: 11.4, p = 0.001, \eta^2 = 0.15$) but not floating time (Treatment: $F_{2,42}: 11.65, p = 0.0001, \eta^2 = 0.34$; Sex: $F_{1,42}: 3.81, p = 0.057, \eta^2 = 0.05$) with no interaction ($F_{2,42}: 0.39, p = 0.67, \eta^2 = 0.01$ and $F_{2,42}: 0.20, p = 0.81, \eta^2 = 0.00$ respectively). No sexual dimorphism or interaction was observed in the percentage of entries (Treatment: $F_{2,42}: 3.92, p = 0.02, \eta^2 = 0.14$; Sex: $F_{1,42}: 2.17, p = 0.14, \eta^2 = 0.04$; Interaction: $F_{1,42}: 0.21, p = 0.53, \eta^2 = 0.00$) or time spent in the open arm (Treatment: $F_{2,42}: 2.98, p = 0.06, \eta^2 = 0.11$; Sex: $F_{1,42}: 1.39, p = 0.24, \eta^2 = 0.02$; Interaction: $F_{1,42}: 0.21, p = 0.80, \eta^2 = 0.00$) of the EPM.

Table 1

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VEH or PRL was injected daily (sc) to pups (from PND1 to PND14), while another group was left undisturbed (CONT). At adulthood (PND80–90), male and female rats from the three groups were subjected to the elevated plus maze test (EPM) to evaluate their anxiety-like behavior. The percentage of entries to and the percentage of time spent in the open arms are shown. MEAN ± SEM. n = 8, ANOVA *p < 0.05 vs CONT (MALES; % ENTRIES OPEN ANOVA $\eta^2 = 0.24, \text{CONT vs VEH d} = 1.93$). VEH: Vehicle, PRL: Prolactin, CONT: Control.

VEH and PRL administration do not increase stress parameters in the neonate

To determine whether PRL or VEH daily injections were stressful enough to alter developmental parameters, we evaluated the body weight and the relative weight of the adrenals at PND15 (Table 2) and analyzed CORT and endogenous PRL levels at PND12. We observed no effect of VEH treatment on any of these parameters; however PRL treatment effect was only observed as an increase (Treatment: $F_{2,21}: 4.59, p = 0.01, \eta^2 = 0.24$) (Table 2). The lack of effect on body weight, relative adrenal weight and CORT levels suggests that pups were not chronically stressed due to daily injections. Ovine PRL administration showed a general effect of group to decrease endogenous PRL concentrations ($F_{2,9}: 4.34, p = 0.047, \eta^2 = 0.49$); however no post-hoc differences were observed, probably due to sample number.

Discussion

Physiological alterations resulting from abnormal activation of hormone and neuropeptide systems during critical periods (i.e., neonate) increase the predisposition for the development of certain diseases in
CONT vs PRL

d
$n = 8$; PRL: Prolactin, $n = 8$; CONT: Control, $n = 8$; FS: Forced swimming test.

We evaluated the effects of increased PRL on the subgranular zone of the hippocampal DG at PND15. In rodents, the hippocampal granule cell layer develops during the first two weeks of life (Schlessinger et al., 1975), making this period a sensitive time window. Ten days after injection, BrdU+ nuclei were mainly detected in the SGL. Stereological analysis showed that PRL treatment increased the volume of the DG granule cell layer, which could be accounted by an increase in neuropil, or of glial cells. This increase, combined with the total number of BrdU+ nuclei, resulted in a reduction in the density of newborn cells, indicating that PRL decreases neurogenesis at PND15. Ten days after injection cell differentiation was not completely achieved, however most of the BrdU+ nuclei co-localized with either DCX or NeuN, indicating their neural nature. No differences in the ratio of double labeled cells were observed among treatments, suggestive that PRL treatment is not affecting the differentiation rate of BrdU+ nuclei. PRL effects might result either from reduced cell proliferation or increased cell death. Fluorojade B analysis revealed no significant effects of PRL treatment on developmental cell death in the DG, although a trend to reduce cell death was observed. This indicates that PRL decreased cell proliferation in the neonate rather than decreasing cell survival.

A significant increase in BrdU+ granular cell density was observed in the OB of VEH pups at PND15 compared with CONT pups that might be accounted as a handling effect. PRL induced a significant decrease of BrdU+ granular cells in the OB compared with VEH, but the number of BrdU+ granular cells in the OB was higher compared to CONT group. PRL stimulates neurogenesis in the SVZ in adult males and females (Shingo et al., 2003). This stimulation enhances olfactory discrimination and regulates emotionality during motherhood (Larsen and Grattan, 2010). Thus, the decrease of neural precursors due to neonatal administration of PRL affects the olfactory function and could possibly contribute to the depressive-like behavior in adulthood.

The mechanisms of PRL action to modulate neurogenesis in the hippocampus and the OB are apparently different. The presence of PRL receptors in the hippocampus has been reported by several groups (Bakowska and Morrell, 1997; Tejadilla et al., 2010; Torner et al., 2009), but not by others (Shingo et al., 2003). However, PRL effects targeting the adult hippocampus are neuroprotective: i.e., PRL administration prevents the stress-induced decrease of neurogenesis (Torner et al., 2009), and protects the hippocampus against kainate-induced excitotoxicity (Tejadilla et al., 2010). PRL stimulates neurogenesis in the SVZ but not the hippocampus (Larsen and Grattan, 2010; Shingo et al., 2003); although it was reported recently that PRL activates a pool of latent precursor cells (Schlessinger et al., 1975), making this period a sensitive time window. Ten days after injection, BrdU+ nuclei were mainly detected in the SGL. Stereological analysis showed that PRL treatment increased the volume of the DG granule cell layer, which could be accounted by an increase in neuropil, or of glial cells. This increase, combined with the total number of BrdU+ nuclei, resulted in a reduction in the density of newborn cells, indicating that PRL decreases neurogenesis at PND15. Ten days after injection cell differentiation was not completely achieved, however most of the BrdU+ nuclei co-localized with either DCX or NeuN, indicating their neural nature. No differences in the ratio of double labeled cells were observed among treatments, suggestive that PRL treatment is not affecting the differentiation rate of BrdU+ nuclei. PRL effects might result either from reduced cell proliferation or increased cell death. Fluorojade B analysis revealed no significant effects of PRL treatment on developmental cell death in the DG, although a trend to reduce cell death was observed. This indicates that PRL decreased cell proliferation in the neonate rather than decreasing cell survival.

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Indirect mechanisms of PRL actions might include generation of circulating vasoactive intestinal peptide (VIP) and vasoactive intestinal peptide (VIP) exerts opposite effects to native PRL and might decrease cell survival.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight (g)</th>
<th>Size (cm)</th>
<th>Adrenals (mg/g BW)</th>
<th>Corticosterone (ng/ml)</th>
<th>rProlactin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>31.1 ± 0.4</td>
<td>16.1 ± 0.17</td>
<td>0.25 ± 0.01</td>
<td>24.49 ± 3.49</td>
<td>1.56 ± 0.04</td>
</tr>
<tr>
<td>VEH</td>
<td>30.7 ± 0.06</td>
<td>16.1 ± 0.08</td>
<td>0.29 ± 0.01</td>
<td>27.07 ± 2.39</td>
<td>1.72 ± 0.36</td>
</tr>
<tr>
<td>PRL</td>
<td>28.8 ± 0.08*</td>
<td>17.0 ± 0.08*</td>
<td>0.25 ± 0.02</td>
<td>21.55 ± 2.66</td>
<td>0.85 ± 0.1</td>
</tr>
</tbody>
</table>

Body weight, body mass, size, and adrenal relative weight of PND15, and CORT plasma levels of PND12 control (CONT) and vehicle (VEH) or prolactin (PRL) treated pups. MEAN ± SEM. n = 8, ANOVA: $\eta^2 = 0.24$, CONT vs PRL $d = -2.25$; PRL CONC: ANOVA $\eta^2 = 0.24$, CONT vs PRL $d = 4.28$; VEH: Vehicle, PRL: Prolactin, CONT: Control.
as it was shown to promote apoptosis in other cell types (Ferraris et al., 2011). Alternatively, PRL might exert both facilitatory and inhibitory effects on neurogenesis as observed with other PRL actions (Drago and Lissandrrello, 2000). Dual effects have also been observed for GC (Gould et al., 1991).

The hippocampus is involved in the modulation of emotionality and has been implicated in the psychopathology of depression. Hippocampal neurogenesis is differentially affected depending on the innate anxiety level, as observed in rats selectively bred for genetic differences in anxiety-related behaviors and stress responsivity (Lucassen et al., 2009). Here PRL treatment induced anxiety-like behavior in males, and a passive coping strategy in male and female adults. Studies with rodent models of adverse early life experiences have found (George et al., 2010) or not (Faure et al., 2007; Lajud et al., 2012) increased anxiety, but they agree on significant depressive-like behavior (George et al., 2010; Lee et al., 2007). Studies in females reported either no alterations or showed sexual dimorphism (Wigger and Neumann, 1999). Further, reduced OB neurogenesis has been observed in a rat model of depression (Yang et al., 2011) and bilateral olfactory bulbectomy induced depressive-like activity in rats (Kelly et al., 1997). The behavioral effects of PRL could be partially associated to the alterations observed in the hippocampus and the OB, because reduced neurogenesis in the neonate caused by adverse early life experience was shown to correlate with a passive coping strategy in adulthood (Lajud et al., 2012), and decreased SVZ neurogenesis and depressive-like behavior were associated to reduced PRL levels during pregnancy (Larsen and Grattan, 2010).

Several studies have suggested that alterations of AVP and CRH expression contribute to the psychophysiological effects observed after early stress exposure (Murgatroyd et al., 2009; Veenema et al., 2006). AVP neurons of the paraventricular nuclei contribute to hippocampal innervation (Zhang and Hernandez, 2012), and stressful events permanently increase AVP (Vega et al., 2010) and AVP1A receptor expression (Lukas et al., 2010), which could alter hippocampal excitability. Postnatal OXT administration results in a dysregulation of HPA axis (Rault et al., 2013); early experiences or differences in mothering behavior also induce changes in the neonate OXT system (Bales et al., 2011), resulting in alteration of social and emotional behaviors. Chronic PRL treatment increases OXT synthesis and basal release of AVP and oxytocin from the hypothalamus (Donner and Neumann, 2009) or the PVN (Vega et al., 2010) in adult rats, and could induce changes in immature OXT or AVP systems. Hence, PRL could be a novel factor that acts like other neuropeptides (CRH, AVP, OXT) to induce programming effects in the neonate and or result in a depressive-like state in adulthood.

Although the PRL dose used is three to ten times smaller than the one reported as protective in the adult (Tejedilla et al., 2010; Torner et al., 2009), we cannot exclude the possibility that differences in the physiology of the neonate could make it a toxic increase. High levels of PRL induce high CORT levels in the adult, increased PRL levels during sensitive postnatal time windows alter brain ontogeny and thereby program for psychopathology later in life.

**Conflict of interests**

The authors declare that there are no competing financial interests in relation to the work described.

**Acknowledgments**

We would like to thank Dr. Alma Lilia Fuentes and Dr. Esperanza Melendez for providing the cryostat. We are grateful with the Confocal Microscopy Unit of the Centro de Estudios Multidisciplinarios en Biotecnología from the UMSNH for allowing us access to the confocal microscope. We also thank Mr. Daniel Mondragón Huerta and Mr. Antonio Prado Galán for technical support and Mr. Juan Gabriel García Gaytán for assistance in animal facilities. Language editing services were provided by Elsevier webshop. Funding was provided by grants to LT from IMSS (FIS/IMSS/PROT/C2007/086), and CONACyT (CB2007/S5109). IMSS and CONACyT had no further role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

**References**


