Prolactin inhibition at the end of lactation programs for a central hypothyroidism in adult rat

Isabela Teixeira Bonomo¹, Patrícia Cristina Lisboa¹, Magna Cottini Fonseca Passos², Simone Bezerra Alves¹, Adelina Martha Reis³ and Egberto Gaspar de Moura¹

¹Departamento de Ciências Fisiológicas - 5o andar, Instituto de Biologia Roberto Alcântara Gomes and ²Departamento de Nutrição Aplicada, Instituto de Nutrição, Universidade do Estado do Rio de Janeiro, Avenida 28 de setembro, 87, Rio de Janeiro, RJ 20551-030, Brazil

³Departamento de Fisiologia e Biofísica, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

(Correspondence should be addressed to E G de Moura; Email: egberto@pq.cnpq.br)

Abstract

Malnutrition during lactation is associated with hypoprolactinemia and failure in milk production. Adult rats whose mothers were malnourished presented higher body weight and serum tri-iodothyronine (T₃). Maternal hypoprolactinemia at the end of lactation caused higher body weight in adult life, suggesting an association between maternal prolactin (PRL) level and programming of the offspring's adult body weight. Here, we studied the consequences of the maternal PRL inhibition at the end of lactation by bromocriptine (BRO) injection, a dopaminergic agonist, upon serum TSH and thyroid hormones, thyroid iodide uptake, liver mitochondrial α -glycerophosphate dehydrogenase (mGPD), liver and pituitary de-iodinase activities (D1 and/or D2), and *in vitro* post-TRH TSH release in the adult offspring. Wistar lactating rats were divided into BRO – injected with 1 mg/twice a day, daily for the last 3 days of lactation, and

C – control, saline-injected with the same frequency. At 180 days of age, the offspring were injected with ¹²⁵I i.p. and after 2 h, they were killed. Adult animals whose mothers were treated with BRO at the end of lactation presented lower serum TSH (–51%), T₃ (–23%), and thyroxine (–21%), lower thyroid ¹²⁵I uptake (–41%), liver mGPD (–55%), and pituitary D2 (–51%) activities, without changes in the *in vitro* post-TRH TSH release. We show that maternal PRL suppression at the end of lactation programs a hypometabolic state in adulthood, in part due to a thyroid hypofunction, caused by a central hypothyroidism, probably due to decreased TRH secretion. We suggest that PRL during lactation can regulate the hypothalamus–pituitary–thyroid axis and programs its function. *Journal of Endocrinology* (2008) **198**, 331–337

Introduction

Some studies have shown that adverse situations that affect the development in critical periods of life, such as undernutrition or hormonal changes, would be able to influence the structure and physiology of organs and tissues in a permanent way (Walker & Courtin 1985, Pracyk *et al.* 1992, Dorner & Plagemann 1994, de Moura & Passos 2005). This biological phenomenon that establishes the relationship between these stimuli in critical periods of life, such as gestation and lactation, and future functional state is called programming (Lucas 1994, Barker 2004, de Moura & Passos 2005).

Lactation is a critical period because in this phase important cognitive and neurological development occurs, which suggests that adverse environmental changes can cause physiological modifications that are able to predispose the development of some diseases in adulthood (de Moura & Passos 2005, Miñana-Solis & Escobar 2006).

We evidenced that adverse situations early in life, such as malnutrition (Passos *et al.* 2002, Teixeira *et al.* 2002, Dutra *et al.* 2003, Passos *et al.* 2004, Vicente *et al.* 2004, Fagundes

et al. 2007, de Moura *et al.* 2007) and hormonal (de Oliveira Cravo *et al.* 2002, Teixeira *et al.* 2003, Lins *et al.* 2005, Toste *et al.* 2006a,b, Bonomo *et al.* 2007, Passos *et al.* 2007) changes during lactation, could permanently affect the progeny. Maternal energy malnutrition during lactation programs for a higher body weight in adulthood; however, protein malnutrition programs for a lower body weight in the adult offspring (Passos *et al.* 2000, Teixeira *et al.* 2002).

We have suggested a relationship between neonatal nutritional status and thyroid function in adult life, because we have demonstrated that maternal protein malnutrition during lactation programs for a hyperthyroidism at adulthood in rodents (Passos *et al.* 2002, Dutra *et al.* 2003, Lisboa *et al.* 2008). Recently, it was reported that women with low birth weight had a higher prevalence of hypothyroidism in adulthood (Kajantie *et al.* 2006). In addition, according to Radetti *et al.* (2006), prematures, independent of their birth weight or length, presented a higher prevalence of disturbance on the hypothalamus–pituitary–thyroid axis later in life.

Maternal malnutrition during lactation is associated with a failure in milk production (Passos *et al.* 2000) in rats, caused by

hypoprolactinemia (Lisboa *et al.* 2006). The bromocriptine (BRO) administration to lactating dams at the end of lactation caused milk production inhibition and neonatal malnutrition, evidenced by the lower body weight of their pups at weaning and important changes in the leptin transfer through the milk and pups' leptinemia (Bonomo *et al.* 2005) that reproduces the leptinemia of the offspring from malnourished dams (Teixeira *et al.* 2002). We also detected that, in adult life, offspring from BRO-treated mothers developed obesity without hyperphagia, suggesting a hypometabolic state, characterized by higher body weight, higher central and total body fat mass, hyperleptinemia and central resistance to the anorectic effect of leptin (Bonomo *et al.* 2007). Also, leptin administration during lactation to the mothers (Passos *et al.* 2007) or to the pups (Teixeira *et al.* 2003, Toste *et al.* 2006a) programs for higher serum tri-iodothyronine (T_3).

So, the present study was designed to evaluate the later repercussion of maternal hypoprolactinemia upon the programming of the thyroid function in the adult offspring.

Materials and Methods

Three-month-old Wistar rats were maintained in a room under a darkness–light cycle (0700–1900 h) and controlled temperature ($25 \pm 1^\circ\text{C}$). Virgin female rats were caged with one male rat at a proportion of 2:1. After mating, each female was placed in an individual cage with water and food made available *ad libitum* until parturition. The use of the animals was according to the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEA/186/2007), which based their analysis on the principles described in the Guide for the Care and Use of Laboratory Animals (Bayne 1996).

Experimental model of maternal hypoprolactinemia during lactation

After birth, excess pups were removed, so that only six male pups were kept per dam, because it has been shown that this procedure maximizes lactation performance (Fischbeck & Rasmussen 1987).

Lactating rats were separated into the following groups: BRO – treated with 1 mg bromo- α -ergocriptine s.c. (BRO – Novartis, São Paulo, Brazil), twice a day, for 3 days at the end of lactation, and C – control group, which received saline for the same time. We used six lactating rats per group and two pups of each dam were randomly separated ($n = 12$ pups).

On day 21 of lactation, dams' serum prolactin (PRL) levels were measured by specific RIA using reagents supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDKD, NIH, Bethesda, MD, USA). Data are reported in ng/ml from the reference preparation, RP-3. Samples were analyzed in a single assay and the intra-assay coefficient was 8%. In addition, we performed an estimate of milk production as described previously (Passos *et al.* 2000, Bonomo *et al.* 2005).

Maternal food intake was measured. We also followed the body weight of mothers and pups during lactation.

After weaning, body weight and food intake were monitored every 4 days until 180 days. At 180 days, in order to determine the thyroid's 2 h radioiodine uptake, the offspring received a single i.p. injection containing 2.22×10^4 Bq of ^{125}I (CNEN, Rio de Janeiro, Brazil). After 2 h, animals were killed by decapitation to collect blood, thyroid, pituitary, and liver.

Thyrotropin (TSH), T_3 , and T_4 serum concentrations

TSH was measured by specific RIA, using a kit for rat TSH supplied by the NIDDKD (NIH) and data were expressed in terms of the reference preparation provided (RP-3). The intra-assay variation coefficient was 7.1%, with 0.09 ng/ml as the lower limit of detection.

Total serum T_3 (TT_3) and thyroxine (TT_4) were determined by RIA, using commercial kits (ICN pharmaceuticals, Inc., Costa Mesa, CA, USA), in which we used control standard curves diluted in iodothyronine-free rat serum (charcoal treated). The intra-assay variation coefficient for T_4 was 5.7%, with 2 $\mu\text{g}/\text{dl}$ as the lower limit of detection, and for T_3 the values were 5.6% and 25 ng/dl respectively.

Mitochondrial GPD activity

The liver α -glycerol-3-phosphate dehydrogenase activity (GPD) was measured in the mitochondrial fraction using phenazine methosulfate (PMS) as an electron transporter between the reduced enzyme and iodonitrotetrazolium chloride violet (INT) (Bernal *et al.* 1978, Oliveira *et al.* 2007). The assay was performed in the presence of 0.1 M DL- α -glycerophosphate diluted in potassium cyanide (KCN)/potassium phosphate buffer (KPB) and a solution of 7.9 mM INT–0.12 mM PMS. Samples were analyzed at 500 nm and the values were expressed as absorbance (O.D)/mg of mitochondrial protein. Protein was measured using the method described by Bradford (1976).

Iodothyronine de-iodinase activity

Type 1 (D1) and 2 (D2) de-iodinase activities were measured based on methods described previously (Dutra *et al.* 2003, Lisboa *et al.* 2003a) by the release of ^{125}I from ^{125}I -reverse T_3 in the liver microsomes and the pituitary total homogenate. Assays were performed in phosphate buffer containing 1 mM EDTA (pH 6.9). D1 assay was performed in the presence of r T_3 (1.5 μM for the liver and 2 nM for the pituitary), dithiothreitol (DTT) (10 mM), and T_4 (100 nM, only for pituitary D2 inhibition). D2 assay was performed with 2 nM r T_3 , 10 mM DTT, and 1 mM 6-n-propyl-2-thiouracil (PTU) (to inhibit pituitary D1). Equal aliquots of ^{125}I -r T_3 (1.07 mCi/ μg – New England Nuclear-Dupont, Boston, MA, USA), purified by paper electrophoresis were placed to each assay tube. Reaction was started by sample addition with the following amount of protein:

70–150 µg for the pituitaries and 12–20 µg for the liver. A blank tube was run in parallel with each assay, containing 50 µl of the substrate solution and 50 µl buffer, which had its values subtracted from enzyme samples. Reactions were performed on a shaking-bath at 37 °C, and stopped after 30 (liver D1) or 60 (pituitary D1 and D2) minutes by the addition of a mixture of 8% BSA and 10 mM PTU, followed by 20% cold trichloroacetic acid. Samples were centrifuged (1500 g, 4 °C, 5 min) and 200 µl of the supernatants were applied to Dowex 50 W-X2 columns (100–200 mesh hydrogen form Bio-Rad). Free ^{125}I , eluted from the column with 10% acetic acid, was measured in a γ -counter. De-iodination percentual in the presence of the enzyme was around 10–20%. The amount of free ^{125}I in blank was generally less than 1–2% of the total radioactivity in the reaction mixture. The specific enzyme activity was expressed by nanomoles or femtomoles of rT_3 de-iodinated/h mg of protein. Protein was measured by the method described by Bradford (1976).

In vitro TRH-stimulated TSH release

Pituitaries of C and BRO groups were quickly dissected out. The anterior pituitary was separated from the posterior pituitary and transected with a longitudinal midline cut. Each anterior hemipituitary was transferred to a tube containing 1 ml Krebs–Ringer-bicarbonate medium (pH 7.4) and incubated at 37 °C in an atmosphere of 95% O_2 –5% CO_2 in a Dubnoff metabolic shaker (50 cycles/min). After preincubation (20 min), the medium was removed and hemipituitaries were resuspended in 1 ml fresh medium. After 1-h incubation, an aliquot was removed for measurement of basal TSH, and thyrotropin-releasing hormone (TRH) (Sigma) was added at final concentration of 50 nM. Then, glands were incubated for 30 min to determine the TSH release in response to TRH. Each hemipituitary was homogenized in phosphate buffer saline (pH 7.6) for measurement of intra-pituitary TSH content (Rettori *et al.* 1992, Moreira *et al.* 1997, Moura *et al.* 2001, Veiga *et al.* 2004, Lisboa *et al.* 2008). Protein content was measured by the Bradford method (Bradford 1976). The medium and pituitary TSH levels were measured by specific RIA.

Statistical analysis

Data are represented as mean $\pm$ S.E.M. Body weight and food intake evolutions were analyzed by two-way ANOVA

Table 2 Food intake and body weight at weaning. Values are given as the mean $\pm$ S.E.M.

	C	BRO
Dam's food intake (g)	45.3 $\pm$ 2.9	40.9 $\pm$ 2.3
Dam's body weight (g)	248.0 $\pm$ 7.1	222.0 $\pm$ 2.4*
Pup's body weight (g)	55.5 $\pm$ 1.3	51.2 $\pm$ 0.6*

* $P < 0.05$. $n = 6$ mothers and 12 pups/group.

followed by Newman–Keuls multiple comparison tests. The statistical significance of TSH and thyroid iodide uptake were determined by the Mann–Whitney test and the other experimental observations by the Student's unpaired t -test, with significance level set at $P < 0.05$.

Results

As expected, BRO-treated mothers showed lower serum PRL at the end of lactation (day 21: -96% , $P < 0.01$), which caused a significant failure in milk production (Table 1). However, milk PRL of BRO dams and serum PRL of the BRO pups were not altered (Table 1). At weaning (Table 2), BRO dams showed lower food ingestion (-25%) and both mothers and pups presented lower body weight (-10 and -7% respectively, $P < 0.01$).

Table 3 shows that 180-day-old animals' mothers that received BRO at the end of lactation presented higher total body weight (10%, $P < 0.05$) without changes in the food intake (g/day), corroborating our previous study (Bonomo *et al.* 2007).

These animals presented lower serum TSH (-51% , $P < 0.001$, Fig. 1), lower thyroid ^{125}I uptake (-41% , $P < 0.05$, Fig. 2), and lower total serum thyroid hormone levels (T_3 : -23% ; $P < 0.05$, Fig. 3A and T_4 : -21% ; $P < 0.05$, Fig. 3B). Liver mitochondrial GPD (mGPD) activity, which is a T_3 -dependent enzyme, was also lower (-55% , $P < 0.05$, Fig. 4).

Pituitary and liver D1 activity was not affected (Fig. 5a and b respectively) in adult animals whose mothers were BRO treated; however, pituitary D2 activity was lower in BRO group (-51% , $P < 0.05$, Fig. 5c). The *in vitro* TSH release after TRH stimulation was similar in both groups (Fig. 6).

Table 1 Prolactin (PRL) concentrations at weaning. Values are given as the mean $\pm$ S.E.M.

	C	BRO
Dam's serum PRL (ng/ml)	14.6 $\pm$ 6.5	0.5 $\pm$ 0.2*
Milk PRL (ng/ml)	104.1 $\pm$ 41.6	97.2 $\pm$ 34.7
Pup's serum PRL (ng/ml)	2.8 $\pm$ 0.5	2.7 $\pm$ 0.7

* $P < 0.05$. $n = 6$ mothers and 12 pups/group.

Table 3 Body weight and food intake of 180-day-old rats' bromocriptine (BRO) and C offspring. Values are given as the mean $\pm$ S.E.M.

	C	BRO
Body weight (g)	397.4 $\pm$ 8.7	438.2 $\pm$ 20.8*
Food intake (g)	17.2 $\pm$ 0.3	17.5 $\pm$ 0.7

* $P < 0.05$. $n = 12$ animals/group.

Figure 1 Serum TSH concentrations of 180-day-old rats whose dams were BRO treated (black bars) or saline treated (white bars) during 3 days of lactation. Values are given as the mean $\pm$ S.E.M.; * $P < 0.05$; $n = 12$ animals/group.

Discussion

We showed that the maternal blockage of serum PRL concentration with BRO, a specific dopamine D2 agonist, caused a calorie restriction to the offspring, since milk production was suppressed. The lower body weight in the BRO-treated mothers reinforces the concept that PRL may play a stimulatory effect on body weight gain during lactation (Fleming 1976). Thus, the BRO treatment was useful not only to address the importance of PRL, but also serves as an experimental model for energy restriction, since both mothers and pups showed a lower body weight at weaning.

Present data from animals whose mothers were BRO treated at the end of lactation reinforces our previous study of higher body weight, increase in total body fat mass, and normal food intake (Bonomo *et al.* 2007), suggesting a hypometabolic state. It is well documented that thyroid hormones exert important role in thermogenesis and basal metabolic rate (Silva 2006), so this hypometabolic state can be related to the lower serum thyroid hormone levels found in these programmed animals.

Figure 2 Thyroid ^{125}I uptake of 180-day-old rats whose dams were BRO treated (black bars) or saline treated (white bars) during 3 days of lactation. Values are given as the mean $\pm$ S.E.M.; * $P < 0.05$; $n = 12$ animals/group.

Figure 3 Total (A) serum T₃ and (B) T₄ of 180-day-old rats whose dams were BRO treated (black bars) or saline treated (white bars) during 3 days of lactation. Values are given as the mean $\pm$ S.E.M.; * $P < 0.05$; $n = 12$ animals/group.

The lower serum thyroid hormone concentrations presented by adult BRO animals can be explained by the lower TSH concentration. As it was already demonstrated that leptin stimulates TRH and TSH production and secretion (Legradi *et al.* 1997, Seoane *et al.* 2000, Ortiga-Carvalho *et al.* 2002), it is possible that the decrease in serum TSH could be secondary to a hypothalamic leptin resistance. The adult offspring whose mothers were BRO treated at the end of lactation presented a central leptin resistance (Bonomo *et al.* 2007), characterized by a lack of leptin's anorexigenic effect.

Figure 4 Liver mGPD activity of 180-day-old rats whose dams were BRO treated (black bars) or saline treated (white bars) during 3 days of lactation. Values are given as the mean $\pm$ S.E.M.; * $P < 0.05$; $n = 12$ animals/group.

Figure 5 (A) Liver D1, (B) pituitary D1 and (C) pituitary D2 activities of 180-day-old rats whose dams were BRO treated (black bars) or saline treated (white bars) during 3 days of lactation. Values are given as the mean $\pm$ S.E.M.; * $P < 0.05$; $n = 12$ animals/group.

The lower thyroid iodide uptake is another thyroid dysfunction caused by the lower TSH level that can impair thyroid hormone biosynthesis.

The lower liver mGPD activity, a specific tissue marker of thyroid function (Coleoni *et al.* 1983, Brown *et al.* 2002), can be explained by the hypothyroidism detected in the adult BRO group, since mGPD is a T_3 -dependent enzyme. This enzyme is responsible for transforming glycerol in dihydroxy-acetone phosphate and for producing reduced equivalents

to the respiratory chain, during lipolysis, and could also contribute to thermogenesis (Lardy *et al.* 1995, Koza *et al.* 1996, Bobyleva *et al.* 2000, dos Santos *et al.* 2003). So, we can suggest that the lower liver mGPD activity could be related to the hypometabolism observed in these animals (Bonomo *et al.* 2007).

Liver D1 activity, another T_3 -dependent enzyme, that is generally lower in hypothyroidism (Bianco & Kim 2006), showed no change in BRO animals. We suggest that the unchanged D1 activity was due, at least in part, to the hyperleptinemia of these animals (Bonomo *et al.* 2007), since it was already described that leptin increases liver D1 activity (Cusin *et al.* 2000, Lisboa *et al.* 2003b). Therefore, the hyperleptinemia could help in maintaining this enzyme activity, despite the lower thyroid hormone levels.

The *in vitro* TSH release after TRH stimulation was increased in a similar way in both groups (C and BRO), suggesting that the lower serum TSH is not caused by a pituitary failure in TSH synthesis and/or secretion. Then, it is possible that neonatal hypoprolactinemia programs for a hypothalamic failure in the TRH production and/or release in adult offspring. Paradoxically, pituitary D2 was lower when a higher activity was expected, since the animals were hypothyroid (Bianco & Kim 2006). The lower pituitary D2 activity in adult BRO animals reinforces the hypothesis that the hypothyroidism observed in this group is associated with a possible TRH-related defect, since it was already shown that pituitary D2 is stimulated by TRH (Kim *et al.* 1998).

In the present study, we suggest some mechanisms induced by the maternal PRL blockage as responsible for the programming of the hypothalamus–pituitary–thyroid axis in adulthood. In general terms, programming occurs through epigenetic mechanisms, such as DNA methylation or histone acetylation, induced by neonatal stressful events (nutritional, hormonal, or environmental) and may lead to an increased risk of metabolic diseases in the adult offspring (de Moura & Passos 2005), e.g. thyroid dysfunctions (Passos *et al.* 2002, Dutra *et al.* 2003). One of the possible imprinting factors that could act as triggering the epigenetic phenomena is the hyperleptinemia, since we already showed a higher leptin transfer and pup's hyperleptinemia at weaning in this same

Figure 6 *In vitro* TSH release before and after TRH stimulation from pituitaries of 180-day-old rats whose dams were BRO treated or saline treated during 3 days of lactation. Values are given as the mean $\pm$ S.E.M.; * $P < 0.05$, $n = 12$ animals/group.

model of PRL inhibition (Bonomo *et al.* 2005). Also, maternal malnutrition is associated with pup's hyperleptinemia at the end of lactation (Teixeira *et al.* 2002) and programs for higher serum T₃ (Teixeira *et al.* 2003, Toste *et al.* 2006a). It is unlikely that BRO transferred through the milk could affect directly the PRL of the pups, since we have not detected any change in pups' PRL. Thus, these explanations may help to understand the mechanism by which the maternal hypoprolactinemia during lactation permanently change the thyroid function. Perhaps this change can turn undernourished children more susceptible to thyroid disorders in adult life (Passos *et al.* 2002, Dutra *et al.* 2003, Lisboa *et al.* 2008), although it deserves epidemiological and prospective studies.

So, for the first time, we demonstrated that maternal PRL inhibition by the treatment with BRO for a short period at the end of lactation programs for hypothyroidism in the adult offspring, which may originate from a central dysfunction, probably caused by lower TRH release. Taken together, our data provide new evidence that PRL changes during lactation play a crucial role in the regulation of body adiposity and thyroid function.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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