Maternal prolactin inhibition at the end of lactation affects learning/memory and anxiety-like behaviors but not novelty-seeking in adult rat progeny

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ABSTRACT

Maternal hypoprolactinemia at the end of lactation in rats reduces milk production and is associated with offspring's malnutrition. Since malnutrition during development is also known to have long lasting effects on cognition and emotion, in the present study we tested the hypothesis that maternal hypoprolactinemia, induced by bromocriptine treatment, at the end of the lactating period affects memory/learning, novelty-seeking and anxiety-like behaviors in adult male Wistar rats using, respectively, the radial arm water maze (RAWM), the hole board (HB) arena and the elevated plus-maze (EPM). We also analyzed serum corticosterone and thyroid hormone levels at postnatal day (PN) 21. Lactating dams were treated with bromocriptine (BRO, 1 mg twice a day, inhibiting prolactin) or saline from PN19 to 21 (the last 3 days of lactation). BRO offspring had hypercorticosteronemia and hypothyroidism at PN21. In the RAWM, reductions in latency observed in CON rats were initially more accentuated than in BRO ones. By the end of the testing period, latencies became similar between groups. No difference was observed between groups regarding the number of nose-pokes in the HB. In the EPM, BRO rats stayed less time in and had fewer entries into the open-arms than CON ones. This pattern of results indicates that maternal bromocriptine treatment at the end of the lactating period results in poorer memory/learning performance and in higher levels of anxiety-like behavior in the adult offspring, demonstrating that even a relatively short period of malnutrition during development can have long lasting detrimental effects regarding cognition and emotion.

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1. Introduction

Epidemiological and experimental studies have associated physiological alterations at adulthood with adverse conditions early in life, such as malnutrition, hormonal changes and other stressful events that are considered imprinting factors (De Moura et al., 2008; Plagemann and Harder, 2005; Ravelli et al., 1976). This phenomenon, which was initially denominated programming (Barker, 2003) and now is also known as developmental plasticity (Gluckman and Hanson, 2007), has been linked to obesity, diabetes, dyslipidemia, cardiovascular diseases (Armitage et al., 2008) and vulnerability to psychopathological disorders such as anxiety and depression (Charney and Bremner, 1999; Darnaudéry and Maccari, 2008). Several studies in animal models and humans have already shown that not only metabolic alterations but also cognitive and behavioral ones can be programmed (Molina et al., 1987; Kar et al., 2008; Fraga-Marques et al., 2009, 2010).

Undernutrition during early life results in permanent impairments in cognitive functions such as executive function, attention, working memory and visual perception (Kar et al., 2008; Levitsky and Strupp, 1995). Furthermore, several studies assessing the effects of breast-feeding have shown that children who are bottle-fed as babies present lower scores on intelligence tests than those who were breastfed later in life (Lucas et al., 1998; Rodgers, 1978). Since women are becoming progressively more active as working force, for many new mothers the return to work following maternity leave is a significant barrier to the continuation of breastfeeding (Galtry, 1997). Thus, experimental models of shortened lactation may be useful to understand the long lasting effects of a reduced period of breastfeeding on the behavioral, hormonal and nutritional statuses of the offspring.

Maternal hypoprolactinemia in rats can be induced by protein malnutrition of dams during lactation and is associated with reduced

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milk production (Lisboa et al., 2006; Passos et al., 2000). Milk production can also be suppressed by treating lactating dams with bromocriptine (BRO) (Shah et al., 1988). Bromocriptine is an agonist of the type 2 dopaminergic receptor that, when administered to the dams, inhibits prolactin (PRL) synthesis in the pituitary gland (Shah et al., 1988). We have previously shown (Bonomo et al., 2005) that the offspring of dams that were BRO-injected during the last 3 days of lactation became malnourished during this period and that this procedure programmed the progeny, at adulthood, for obesity, hyperleptinemia, resistance to the anorexigenic effects of leptin (Bonomo et al., 2007), hypothyroidism (Bonomo et al., 2008), dyslipidemia, insulin resistance, hypercorticosteronemia and higher total catecholamine in adrenal medulla (De Moura et al., 2009). In spite of the fact that a considerable amount of information is available regarding metabolic and hormonal programming as a result of malnutrition at the end of lactation, none has been provided regarding the function of the nervous system. In this regard, the aim of the present study was to evaluate whether maternal PRL inhibition by the administration of BRO to dams during the late lactating period (from postnatal day 19 to 21) also results in behavioral changes at adulthood by assessing memory/learning, novelty-seeking and anxiety-like behaviors in male adult rat offspring.

2. Methods

2.1. Animal treatment

All experiments were carried out under institutional approval (CEA/187/2007) in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* (Publication no. 85-23, revised 1985) as adopted and promulgated by the National Institutes of Health and in accordance with Brazilian Law (Lei Arouca, Law #11.794, October 8th, 2008) (Marques et al., 2009).

Adult Wistar rats were kept in a temperature-controlled room on a 12-h light/12-h dark cycle (lights on at 7:00 am). Three-month-old virgin female rats were placed with male rats in a 2:1 ratio. After mating, pregnant rats were placed in individual cages. Access to food and water was *ad libitum*. At birth, (postnatal day 1 — PN1), all litters were culled to six male pups so as to maximize lactation performance (Fischbeck and Rasmussen, 1987; Passos et al., 2000). Litters that did not have at least six male pups were discarded. The anogenital distance was used to differentiate females from males and all experimental animals were accurately sexed. Sixteen lactating rats were separated into the following groups: 1) BRO — i.p. injected with 1 mg bromo-*a*-ergocriptine (Novartis, SP, Brazil), twice a day, during the last 3 days of lactation (PN19 to PN21) or 2) CON — which received i.p. injections of saline during the same period (Bonomo et al., 2005). After weaning (PN21), siblings were kept together throughout the experimental period (cage size: 39 cm long × 32 cm wide × 14 cm high) since single housing may influence behavior (Valzelli and Garattini, 1972). BRO and CON offspring had access to commercial diet and water *ad libitum*.

2.2. Body weight

During lactation, the body weights of the offspring were measured at PN1, PN4, PN8, PN12, PN16, PN18, PN19, PN20 and PN21. After PN21, body weight was monitored every fourth day until PN180.

2.3. Hormones

Serum corticosterone level was measured in 4 offspring (selected from different litters) and 5 dams of each group at PN21. After decapitation, which was carried out between 9:00 to 12:00 am, blood samples were collected and centrifuged ($1000 \times g$, 4 °C, 20 min) to obtain serum, which was individually kept at −20 °C. All measure-

ments were performed in one assay. Serum corticosterone level was measured by specific murine RIA kit (ImmuChemTM 125I, double antibody, ICN Biomedicals, Inc, USA). The assay sensitivity was 25 ng/ml and the intra-assay variation was 7.1%.

Total serum triiodothyronine (T3), thyroxine (T4) and TSH were determined by RIA in 8 BRO and 8 CON offspring (selected from different litters) at PN21. We used commercial kits (ICN pharmaceuticals, Inc., Costa Mesa, CA, USA) in which control standard curves diluted in iodothyronine-free rat serum (charcoal treated) were used. The intra-assay variation coefficient for T3 was 5.3%, with 25 ng/dl as the lower limit of detection, and for T4 the values were 3.3% and 2 μg/dl respectively. Serum TSH levels were measured using a kit for rat TSH supplied by the NIH (Boston, MA, USA) and data were expressed in terms of the reference preparation provided (RP-3). The intra-assay variation was 0.6% and the sensitivity limit was 0.18 ng/ml.

2.4. Behavioral testing

From PN172 to PN184, 40 BRO and 37 CON rats underwent behavioral testing. The following tests were used: 1) radial arm water maze (RAWM); 2) hole board arena (HB); and 3) elevated plus-maze (EPM). All testing sessions were performed between 2:00 and 6:00 pm in a sound attenuated room. A CON testing was always followed by a BRO one and *vice versa*. All tests were videotaped and the behavior was analyzed using the video images of the tests.

Memory/learning was assessed by testing rats at PN172 in the RAWM, which is shaped like an asterisk and consists of eight swim paths (arms: 29 cm long × 13 cm wide × 40 cm high) extending out of an open central area (41 cm diameter). The RAWM was filled with water (26 ± 1 °C) to a depth of 34 cm so that rats could not touch their feet or tails while swimming on the surface. An escape platform (8 cm long × 10 cm wide) was placed at the end of one of the arms, 1 cm below the surface. Non-toxic white paint was used to make the water opaque, hiding the platform. Several extra-maze cues were present in the testing room and their positions remained fixed throughout the entire experiment. A test began by placing the animal facing away from the arm that had the escape platform. Animals were tested for 5 consecutive days, 4 trials per day (inter-trial interval: 15 min). Animals were allowed 2 min per trial to explore the maze and find the hidden platform. If they failed to find the platform in a given trial during their allotted time, they were gently guided to it and allowed to stay on top of it for 20 s. The escape platform was not moved from its initial position during the first 4 days. In a sample of 28 BRO and 19 CON offspring, we also carried out a probe trial on the 5th day: The platform was placed in the arm opposite to the one in which it was located during the previous 4 days. The following variables were used to assess performance in the RAWM (Fraga-Marques et al., 2009, 2010; Jarrard, 1993): 1) Latency to find the hidden platform on each trial of each testing day (LAT); 2) Latency to first arm entry (LATARM; 3) Number of first entries into any arm which didn't have the platform (reference memory — RM); 4) Number of re-entries into the arm that had the platform (working memory correct errors — WMC); 5) Number of re-entries into any arm that did not have the platform (working memory incorrect errors — WMI). The pool was cleaned (bedding residue and fecal boli were removed) and the water thoroughly mixed between tests. The water was completely changed every day.

Novelty-seeking behavior was assessed by testing rats at PN180-181 in HB, which has the shape of a rectangular enclosed arena (80 cm long × 60 cm wide × 40 cm high). The base of the arena has 16 uniformly spaced holes (4 cm diameter) that the animals can explore. Animals were placed in the arena facing one of the walls and were allowed 10 min to explore. The number of nose-pokes (head-dips) was used as a measure of exploring activity (Abreu-

Villaça et al., 2006; Fraga-Marques et al., 2009, 2010). The number of rearings and grooming events (both indicating motor activity) was also used as ethological measures (Carola et al., 2002). The arena was cleaned with paper towels soaked in 50% ethanol and dried before each test.

Anxiety-like behavior was assessed by testing rats at PN182–184 in the EPM, which is shaped like a plus sign and consists of two "open" (no walls, 50 cm long×10 cm wide) and two "closed" (50 cm long×10 cm wide×40 cm high) arms (OA and CA respectively), arranged perpendicularly, and elevated 50 cm above the floor. The test began with the animal being placed on the center of the equipment facing an open arm. Each test lasted 10 min. The total time spent in and the number of entries into each kind of arm and central area of the maze was recorded. The percentage of time spent in the open arms (%Time OA: the time spent in open arms divided by time spent in open + closed arms) and the percentage of open arms entries (%Entries OA: the number of entries in open arms divided by number of entries in open + closed arms) was also calculated (Manhães et al., 2008; Fraga-Marques et al., 2009, 2010). The number of closed arms entries (Entries CA) was used as a measure of activity. An entry was counted whenever the animal crossed with all four paws into an arm or into the central area. Increased Time OA, %Time OA, Entries OA or %Entries OA are indicative of a reduced anxiety state in the EPM (Fraga-Marques et al., 2009, 2010; Handley and McBlane, 1993). In addition, the percentage of time spent in the center of the maze (%Time CN: time spent in center divided by total time) was used as an independent measure of decision making (Manhães et al., 2008). Furthermore, ethologically derived parameters were also assessed in the EPM. These included rearing, number of closed arm returns, head dipping, grooming, and stretched attend posture (Johnson and Rodgers, 1996). The EPM was cleaned with paper towels soaked in 50% ethanol and dried before each trial.

2.5. Data analysis

Kolmogorov–Smirnov one sample tests (K–S) were used to assess the normality of the distributions of each of the variables. Significance is assumed at the level of P<0.05. For parametric distributions, data are compiled as means and standard errors of the means, while non-parametric data are compiled as medians and quartiles. A repeated measures analysis of variance (rANOVA) was used to analyze body weight throughout the experiment. TREATMENT (bromocriptine or control) was used as the between-subjects factor and DAY was considered the within-subjects factor. Student *t*-tests were used to compare serum corticosterone, T3, T4 and TSH levels between groups. Differences between the CON and BRO groups regarding latency to find the hidden platform (LAT) in the RAWM were analyzed by means of a repeated measures analysis of variance (rANOVA). DAY and TRIAL were considered the within-subjects factors. TREATMENT (bromocriptine or control) was used as the between-subjects factor. As for the other RAWM variables, LATARM, RM and WMI were analyzed using a multivariate ANOVA (mANOVA), while the WMC was analyzed using the Mann–Whitney test (M–W). The number of nose pokes in the HB was analyzed by means of a rANOVA (INTERVAL: within-subjects factor; TREATMENT: between-subjects factor). HB ethological data were analyzed by means of a multivariate ANOVA (mANOVA–TREATMENT: between-subjects factor). The EPM data Entries OA, %Entries OA, Time OA, and %Time OA were analyzed by means of the Mann–Whitney Test (M–W). Entries OA + CA, %Time CN, Return to CA and the other ethological data were analyzed by means of a mANOVA. (TREATMENT: between-subjects factor). Whenever the sphericity assumptions appeared to be violated (Mauchly's test) in the rANOVAs, an adjustment to the numerator and denominator degrees of freedom was made by using parameter ε (Huynh and Feldt, 1976). Lower-order ANOVAs and the Fisher Protected Least Square Difference test were used post hoc.

3. Results

3.1. Body weight

As indicated in Table 1, during the period of maternal bromocriptine treatment (PN19–PN21), BRO offspring presented significantly reduced body weight (7.7% reduction at PN21 for example) when compared to CON ones. After the treatment period, no significant differences were observed between groups up to the 3rd postnatal month. At PN180, BRO offspring were significantly heavier (+9.3%) than CON ones.

3.2. Hormones

Corticosterone levels of BRO dams at the end of lactation (PN21) were not significantly different than that of CON ones (*t*-test: P>0.10; BRO: 188 ± 32 ng/ml, CON: 278 ± 40 ng/ml). However, BRO offspring had significantly higher serum corticosterone levels than CON ones (*t*-test: P=0.023; BRO: 191 ± 19 ng/ml; CON: 88 ± 23 ng/ml). T3 levels were significantly reduced in BRO animals when compared to CON ones (*t*-test: P=0.004; BRO: 70 ± 3 ng/dl; CON: 86 ± 10 ng/dl), while no differences were observed between groups regarding T4 levels (*t*-test: P>0.10; BRO: 3.3 ± 0.8 µg/dl; CON: 3.3 ± 0.6 µg/dl). BRO animals also had significantly lower TSH levels than CON ones (*t*-test: P=0.016; BRO: 0.24 ± 0.02 ng/ml; CON: 0.32 ± 0.01 ng/ml).

3.3. Behavior

Fig. 1A shows that BRO animals presented a less accentuated improvement in performance (LAT) from the 1st to the 4th day than CON ones (rANOVA–TREATMENT×DAY interaction: P=0.001). This finding can be explained by the fact that while a significant reduction in latency can be observed from the 1st to the 2nd day in CON rats (FPLSD: P=0.002), no differences were observed in BRO ones. Furthermore, the fact that BRO rats were significantly faster (mANOVA: P=0.028) than CON ones in the 1st day and slower (mANOVA: P=0.026) in the 2nd helps to explain the observed interaction. No differences were observed regarding the 3rd, 4th and 5th (probe trial) days. As expected, the change in position of the platform resulted in increased latencies in both groups (FPLSD—4th *vs.* 5th day: P<0.001). The observed differences in latency in the 1st and 2nd days are associated with differences in RM (Fig. 1C) and WMI errors (Fig. 1D). In both cases, BRO animals presented a better performance than CON ones in the 1st day, and a worst one in the second. No differences were observed between groups regarding LATARM (irrespective of the position of the platform) (Fig. 1B) and the number of WMC errors. In the latter case, the median for both groups was zero with an interquartile range also of zero.

Maternal hypoprolactinemia had no effect (rANOVA: P>0.10) on the total number of nose pokes made by the adult offspring in the HB, as indicated in Fig. 2A. However, during the first time interval (initial 2.5 min), BRO animals had a tendency (rANOVA: P=0.055) to make less nose pokes than CON ones (Fig. 2B). No differences between groups were observed regarding the number of grooming (Fig. 2C) and rearing events (Fig. 2D).

As depicted in Fig. 3, BRO rats presented significantly reduced absolute and relative time spent in the open arms (M–W—Time OA: P=0.015; %Time OA: P=0.009, respectively) as well as a significant reduction in absolute number of entries into the open arms (M–W—Entries OA: P=0.021) when compared to the CON ones (Fig. 3A–C). BRO rats also presented reduced: Entries CA (mANOVA: P<0.001; Fig. 3F) and %Time Center (mANOVA: P=0.002; Fig. 3E). Regarding the ethological data, the only measure for which a significant difference was observed was the Closed Arm Returns (mANOVA: P=0.002).

Table 1
Body weight of the offspring throughout the experiment. Postnatal day 19 is the first day of bromocriptine treatment.

Group	Postnatal day										
	0	8	16	18	19	20	21	45	89	133	180
Control	6.8 ± 0.1	20.4 ± 0.3	38.3 ± 0.8	46.9 ± 1.0	50.4 ± 1.1 PS	53.1 ± 1.1[*]	55.5 ± 1.3[**]	183.7 ± 6.0	326.7 ± 7.9	377.5 ± 8.1[***]	397.4 ± 8.9[***]
Bromocriptine	6.5 ± 0.1	20.4 ± 0.6	38.3 ± 0.4	45.0 ± 0.7	47.9 ± 0.8	50.1 ± 0.7	51.2 ± 0.6	196.1 ± 4.0	345.8 ± 11.3	414.3 ± 16.4	438.2 ± 20.9

Values are means ± S.E.M, PS 0.10 ≥ P ≥ 0.05.
  [*] P < 0.05.
 [**] P < 0.01.
[***] P < 0.001.

4. Discussion

4.1. Maternal bromocriptine treatment and changes in offspring's body weight and hormonal levels

In a previous paper (De Moura et al., 2009), we described somatic, metabolic and hormonal changes at different ages of the siblings of rats used in the present study, as well as data concerning serum PRL levels of the dams at weaning. We demonstrated that bromocriptine treatment significantly reduced PRL levels in the dams (−95%), but did not affect offspring levels at PN21, as expected (Bonomo et al., 2005; De Moura et al., 2009). In the present study, we confirm previous results (De Moura et al., 2009) indicating that treatment of dams with bromocriptine has deleterious effects on body weight gain of the offspring from PN19 to PN21, in spite of the fact that solid foods are already found in the offspring stomach during this period (Gomendio et al., 1995). It was also confirmed that BRO rats became heavier than CON ones at adulthood.

In general, fasting increases glucocorticoid levels (Ahima et al., 1996), which is consistent with our observation of higher corticosterone levels in BRO pups at PN21. This higher concentration in the offspring, but not in the dams, would seem to indicate that the former were stressed by the period of malnourishment while the dams were relatively unaffected by treatment with bromocriptine. Corticosterone is also known to affect thyroid function at several levels. For example, it inhibits the TRH–TSH–thyroid axis and the type 1 deiodinase in the liver, which converts T4 to T3 (Haugen, 2009). Furthermore, fasting also directly affects serum thyroid hormone concentrations (Moura et al., 1987). The aforementioned findings are consistent with our observations of lowered serum TSH and T3 levels. Interestingly, T4 levels did not vary as a function of the bromocriptine treatment. In explaining this result, one has to keep in mind that: 1) T4 has a large intrathyroidal pool (Chanoine et al., 1993); 2) T4 half-life in the serum is considerably longer than that of T3 (Unger et al., 1980); and 3) The T4–T3 conversion is inhibited (Johnson et al., 2003). Therefore, it is conceivable to speculate that a
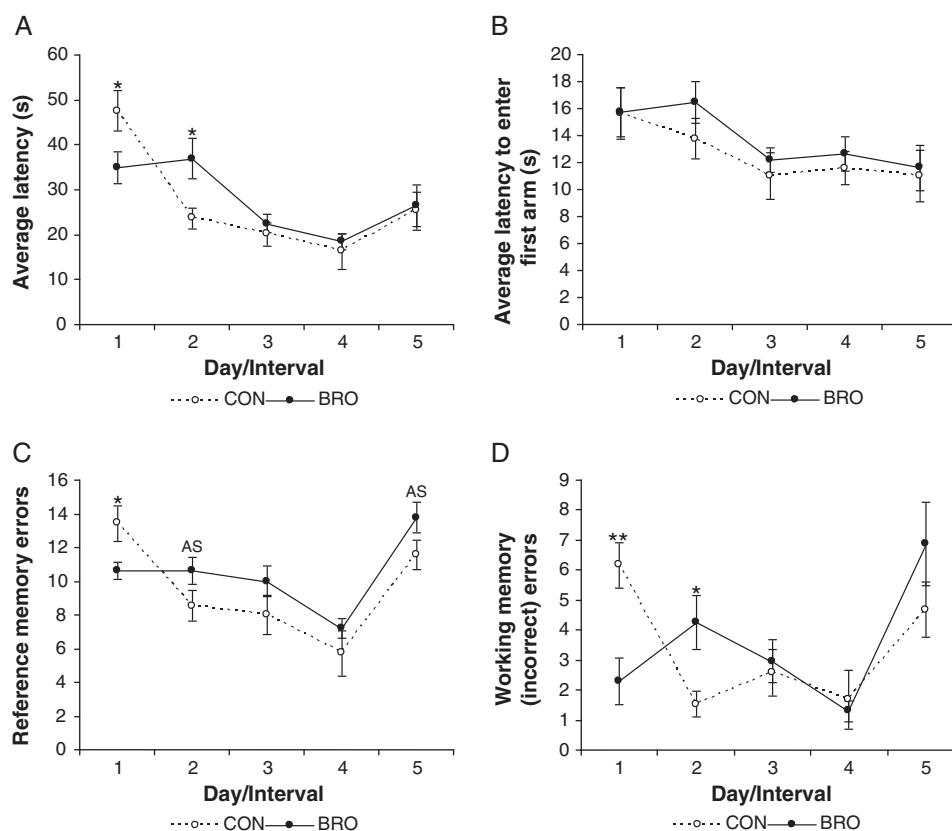

Fig. 1. Memory/learning performance as measured in the radial arm water maze. BRO animals presented a much less accentuated improvement in performance from the 1st to the 4th testing day (A). This result is associated with a higher number of reference memory errors (C) and higher number of working memory (incorrect) errors (D), but does not seem to be related with the latency to enter the first arm (B). Values are means ± SEM. AS 0.10 < P < 0.05, *P < 0.05, and **P < 0.01.
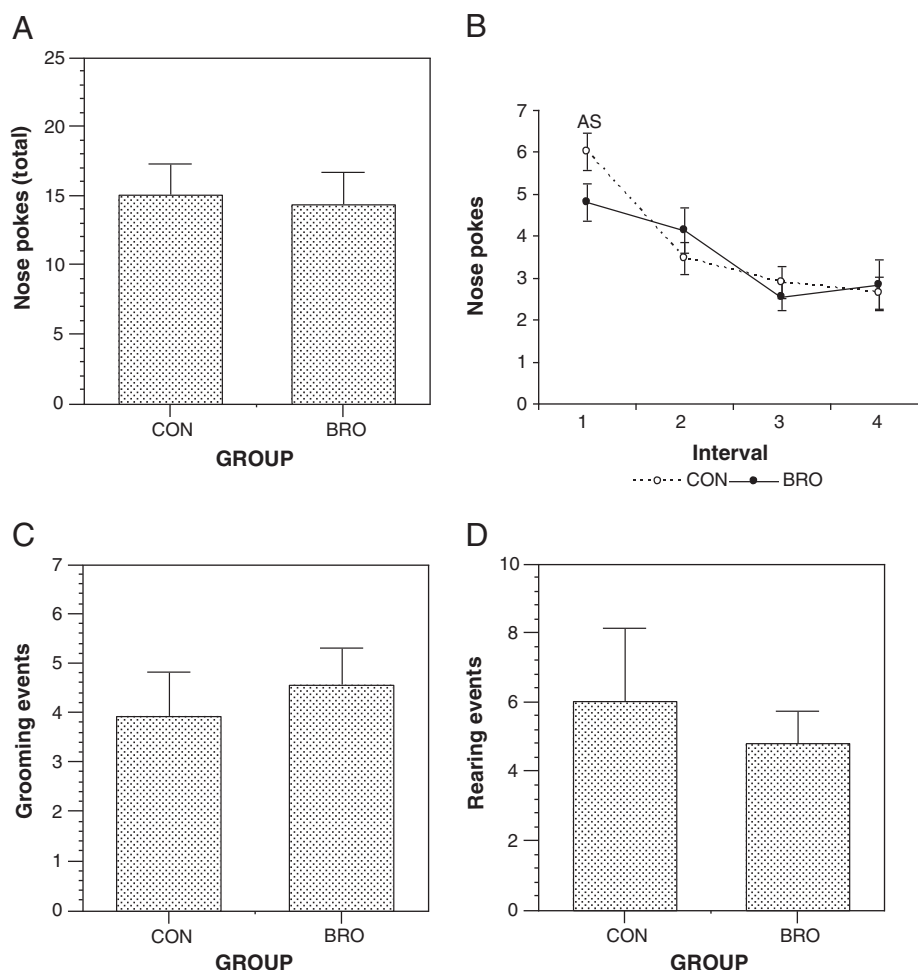
Fig. 2. Novelty-seeking behavior as measured by the number of nose pokes in the hole board (HB) arena. No difference was observed between bromocriptine (BRO) and control (CON) rats regarding the total number of nose pokes (A). When intra-session intervals were considered, a difference approaching significance was observed for the first interval (P = 0.055 — B). Motor activity, as measured by the number of grooming (C) and rearing (D) events in the HB, was also not affected by BRO treatment. Values are means ± SEM. AS 0.10 < P < 0.05.

longer period of malnourishment would be needed to cause a detectable reduction in serum T4 levels.

4.2. Behavior

Our main findings indicate that maternal hypoprolactinemia induced by bromocriptine treatment during the late lactating period causes behavioral changes in the adult offspring. In the radial arm water maze, BRO rats had a less pronounced gain in performance from the 1st to the 4th testing day than CON ones, indicating that the former had significant alterations in visuospatial memory/learning mechanisms. In spite of the fact that the performance gain was more evident in CON animals, a peculiar finding was that BRO offspring were faster than CON ones in locating the escape platform during the 1st testing day. The latencies to enter for the first time any of the arms were equivalent in both groups throughout the testing sessions; therefore performance differences in the first testing day cannot be attributed to a delay in the decision to start moving and exploring the maze. Furthermore, the number of working memory errors of the correct type (as measured by the number of re-entries into the arm in which the platform was located) was very low and similar between groups, indicating that most animals, irrespective of treatment, upon entering the correct arm almost always found the platform and successfully exited the water. On the other hand, during the 1st day, BRO animals made significantly less reference memory errors (−21%, as measured by the number of first entries into arms that did not have

the platform) and considerably less working memory errors of the incorrect type (−63%, as measured by the number of re-entries into arms that did not have the platform) than CON rats. This pattern of results suggests that the working memory of BRO rats is not as affected as that of CON rats by the initial contact with the RAWM, leading to a shorter path length to find the platform and the consequent reduced latency. By the second testing day, the initial BRO advantage is lost mainly due to a marked reduction in working memory errors of the incorrect type by CON rats, an improvement in performance that was completely absent in BRO offspring.

As for the EPM, our results indicated that BRO offspring displayed more intense anxious-like behavior, characterized by a shorter time spent in and reduced number of entries into the open arms. These animals also spent less time in the central area of the EPM, which has been associated with decision making processes (Johnson and Rodgers, 1996) and had a reduced number of closed arm entries, indicative of lowered overall locomotor activity. No differences were observed regarding the ethologically derived measures associated with risk assessment, vertical activity and exploration (Rodgers et al., 1997), suggesting that the effects of BRO programming on behavior were selective.

Both groups displayed a similar level of novelty-seeking behavior in the HB. Furthermore, the analyses of the number of grooming and rearing events in the HB also failed to indicate any differences between groups. The absence of differences between groups regarding these behavioral traits in the HB gives further support to the
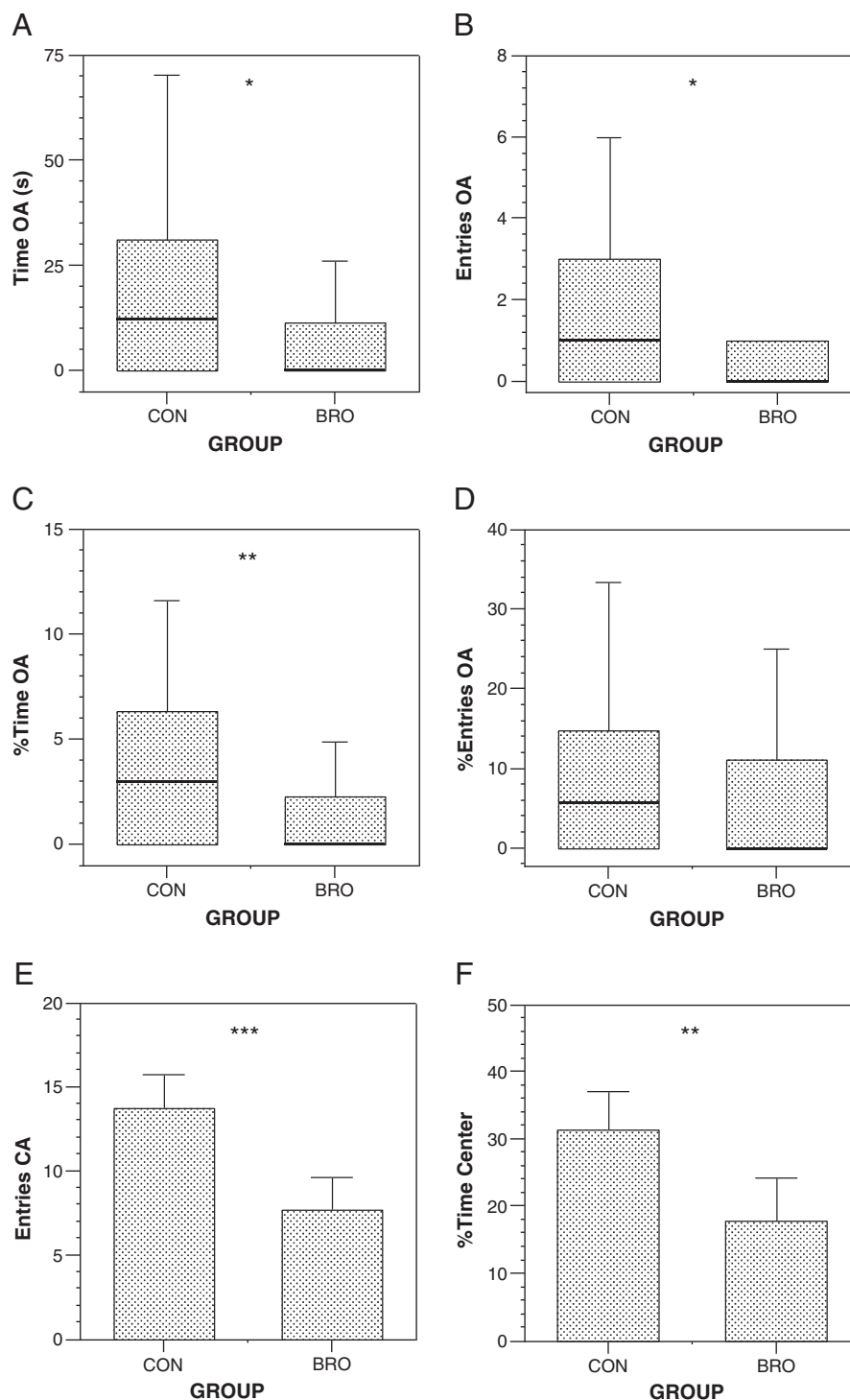
Fig. 3. Anxiety-like behavior tested in the elevated plus maze. Bromocriptine (BRO) rats had significantly lower Time OA (A), Entries OA (B) and %Time OA (C) than control (CON) ones, indicative of higher anxiety levels. Locomotor activity (Entries CA − E) and decision making (%Time Center − F) were also reduced in BRO animals. A, B, C, and D: values are medians ± quartile, E and F: values are means ± SEM, *P<0.05, **P<0.01, and ***P<0.001.

notion that the effects of BRO programming on behavior were selective.

In summary, a three-day period of malnutrition resulting from the inhibition of milk production by bromocriptine treatment of dams at the end of lactation affected learning and memory mechanisms and increased anxiety-like behavioral traits in adult Wistar rats without modifying novelty-seeking behavior. In addressing possible explanations for the present results, two lines of reasoning, which are not mutually exclusive, may be used: 1) the altered behaviors were

directly programmed during the period of malnourishment at the end of lactation, and/or 2) the behavioral changes are a consequence of the metabolic/hormonal changes programmed during the lactation period.

4.3. Malnutrition, brain development and behavior

Events that affect the central nervous system (CNS) during sensitive periods of its development may result in long-lasting

changes in the formation and function of brain circuits (Bhatnagar et al., 2005; Charney and Bremner, 1999) and could increase the chance of developing cognitive deficits at adulthood (Charney and Bremner, 1999; Strupp and Levitsky, 1995). Malnourished children have profound changes on brain development such as disorderly differentiation, decreased number of synapses and synaptic neurotransmitter content, delayed myelination and reduced overall development of dendritic arborization (Udani, 1992; Wang and Brand-Miller, 2003). The important changes in the temporal sequence of brain maturation associated with malnutrition permanently impair cognitive functions (Strupp and Levitsky, 1995). For instance, cognitive flexibility, attention, working memory, visual perception and memory performance are affected by child malnutrition (Kar et al., 2008).

In rodents, a period of rapid brain growth begins prenatally, mainly during the last week of gestation, and peaks during lactation (Andrade et al., 1996). There are studies in rats indicating that the postnatal period should be susceptible to the deleterious effects of malnutrition on brain function because of its rapid growth during this period (Dobbing, 1964; Winick and Noble, 1966). In particular, the first 3 weeks of postnatal life correspond to a time-window of intense hippocampal and hypothalamic neuronal circuit development (Bouret et al., 2004; Coupé et al., 2009), suggesting that early perinatal modifications may have long-lasting consequences on behavior and neuroendocrine parameters (Bunsey and Eichenbaum, 1995, 1996). In fact, animal studies have shown that early malnutrition permanently affects brain structure (Jahnke and Bedi, 2007; Morgane et al., 1993; Randt and Derby, 1973) as well as behavior (Levay et al., 2008; Pereira-da-Silva et al., 2009). Alterations in hippocampal development and function as a result of malnutrition (Jahnke and Bedi, 2007; Levitsky and Strupp, 1995) are consistent with our current findings of impaired performance gain in the RAWM and higher anxiety levels in the EPM displayed by BRO rats, since the hippocampus has an important role in normal memory processing (O'Keefe, 1990; Olton et al., 1978), locomotor activity and anxiety-like behavior (Bannerman et al., 2004; Bast and Feldon, 2003). Compounding the effects of malnutrition on the structural development of the hippocampus is the fact that this structure is also particularly sensitive to anxiogenic factors (Ciaroni et al., 2002). Malnutrition is a particularly stressful event (Yehuda et al., 2009), an observation that is in agreement with our finding of increased corticosterone levels in BRO animals at PN21. Malnourishment is not only a stressful factor that affects learning/performance but also programs for anxiety (Levitsky and Barnes, 1970) and changes in decision making (Taylor and MacQueen, 2007). Our results complement previous findings in the sense that we demonstrated that even restricted periods of malnourishment resulting from a premature interruption of the lactation period has long lasting behavioral effects.

Considering that bromocriptine is an agonist of the type 2 dopaminergic receptor, it is not inconceivable that this substance could have affected the dams' maternal behavior, which, in turn, could have had long lasting effects on offspring behavior. Bromocriptine could affect maternal behavior directly, by altering dopaminergic function (Koller, 1992), or indirectly via reduction of PRL levels. PRL is associated with the onset of maternal behavior in rats (Mann and Bridges, 2001). Furthermore, PRL can have antidepressive and anxiolytic effects, reducing HPA axis responses to stress in the rat (Drago et al., 1990; Frye and Walf, 2004; Lund et al., 2005; Torner et al., 2002). Since bromocriptine treatment did not have a significant effect on the dams' corticosterone levels and since PRL has not been implicated in the continuation of maternal behavior, particularly at the end of the lactation period, an explanation for our results based on behavioral alterations in the dams is rendered unlikely.

Early stressors may have a permanent rather than transient effect on the organism (Darnaudéry and Maccari, 2008). Early stress leads to long-term consequences from both behavioral and neurobiological points of view, increasing susceptibility to anxiety- and depression-like behavior and memory impairment in the offspring (Charney and Bremner, 1999; Darnaudéry and Maccari, 2008; Vallée et al., 1999). Among the mechanisms involved in these behavioral alterations are increased responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to stress and the endocrine stress response (Henry et al., 1994; Morley-Fletcher et al., 2003a,b; Vallée et al., 1997), which is accompanied by reduced hippocampal plasticity (Darnaudéry and Maccari, 2008). During neonatal life, psychological stress has been associated with an increase in corticotropin releasing hormone (CRH) expression in the amygdala and hippocampus (Hatalski et al., 2000), which also may exert a role in the long-lasting effects of early-life stress (Brunson et al., 2001).

Thyroid hormones also affect several processes in brain development during different time windows (Anderson, 2001; Bernal, 2002; Zoeller and Rovet, 2004). Experimental studies in rat have firmly established that thyroid deficiency during the early postnatal period markedly impairs the development of the CNS and delays the maturation of innate reflexes and behavior (Eayrs, 1964; Legrand, 1983; Meisami et al., 1992). Some of the most prominent actions of thyroid hormones occur on 2nd and 3rd week postnatal (Dussault and Ruel, 1987; Porterfield and Hendrich, 1993; Thompson and Potter, 2000). In the rat, several aspects of adaptive, cognitive and motor development, which are believed to be related to hippocampal development (Korányi et al., 1976; Leblanc and Bland, 1979), can be impaired in the presence of postnatal hypothyroidism (Tamasy et al., 1986a,b; Hasegawa et al., 2010).

4.4. Endocrine parameters at adulthood and behavior

The programming model used in the present study has several characteristics that can increase the complexity of the discussion of our results. For instance, we have previously demonstrated that BRO offspring, as adults, present higher central adiposity, higher triglycerides, lower HDL-c, insulin resistance (De Moura et al., 2009), hypothyroidism (Bonomo et al., 2008), hypercorticosteronemia, higher adrenal catecholamine content (De Moura et al., 2009), hyperleptinemia and resistance to the anorexigenic effect of leptin (Bonomo et al., 2007). These characteristics, by themselves or in conjunction, are known to interfere with behavior (Farr et al., 2008; Fraga-Marques et al., 2009, 2010; Harvey and Ashford, 2003; Kamal et al., 1999).

Cognitive deficits have already been evidenced in obese rodents with associated hypertriglyceridemia (Farr et al., 2008) and rodents with diabetes (Harvey and Ashford, 2003; Kamal et al., 1999). As another example, we have recently shown that rats that were programmed for hyperleptinemia and resistance to the anorexigenic effect of leptin at adulthood had significant behavioral alteration in the RAWM, EPM and HB (Fraga-Marques et al., 2009; 2010). Concerning the effects of elevated corticosterone levels at adulthood, it has been shown that infusion of glucocorticoids into the amygdala can increase anxiety and also further increase glucocorticoids secretion (Myers and Greenwood-Van Meerveld, 2007; Shepard et al., 2000, 2003). We have also previously shown that rats with higher serum corticosterone levels at adulthood display increased anxiety-like behavior in the EPM and impaired learning/memory performance in the RAWM (Fraga-Marques et al., 2009). As for hypothyroidism at adulthood, several studies have already shown that the thyroid status is linked with anxiety-like behavior and impaired learning in rodents (Guadaño-Ferraz et al., 2003; Pilhatsch et al., 2010; Venero et al., 2005) and more specifically with decreased locomotor activity in the EPM (Fundaro, 1989; Redei et al., 2001).

5. Conclusions

Our data indicate that milk inhibition at the end of lactation caused by maternal treatment with bromocriptine results in hypercorticosteronemia and hypothyroidism at the end of the period of restricted

nutrition. We further demonstrated that bromocriptine treatment resulted in altered learning/memory performance and higher levels of anxiety-like behavior, without interfering with novelty-seeking behavior, in the adult offspring. Subsequent studies will have to address whether corrected to normal parameters regarding HPA axis and thyroid status can improve the observed behavioral alterations or if behavioral modifications are a permanent effect of altered brain development and function resulting from early malnutrition. The present study suggests that the experimental model used here will be useful to address the effects of shortened periods of lactation, a condition that is becoming increasingly more prevalent in modern society.

Acknowledgments

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