Early weaning by maternal prolactin inhibition leads to higher neuropeptide Y and astrogliosis in the hypothalamus of the adult rat offspring

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Abstract

The suppression of prolactin production with bromocriptine (BRO) in the last 3 d of lactation reduces milk yield (early weaning) and increases the transfer of leptin through the milk, causing hyperleptinaemia in pups. In adulthood, several changes occur in the offspring as a result of metabolic programming, including overweight, higher visceral fat mass, hypothyroidism, hyperglycaemia, insulin resistance, hyperleptinaemia and central leptin resistance. In the present study, we investigated whether overweight rats programmed by early weaning with maternal BRO treatment have hypothalamic alterations in adulthood. We analysed the expression of neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART), pro-opiomelanocortin (POMC) and α-melanocyte-stimulating hormone (α-MSH) by immunohistochemistry in the following hypothalamic nuclei: medial and lateral arcuate nucleus (ARC); paraventricular nucleus (PVN); lateral hypothalamus (LH). Additionally, we sought to determine whether these programmed rats exhibited hypothalamic inflammation as indicated by astrogliosis. NPY immunostaining showed a denser NPY-positive fibre network in the ARC and PVN (+82 % in both nuclei) of BRO offspring. Regarding the anorexigenic neuropeptides, no difference was found for CART, POMC and α-MSH. The number of astrocytes was higher in all the nuclei of BRO rats. The fibre density of glial fibrillary acidic protein was also increased in both medial and lateral ARC (6·06-fold increase and 9·13-fold increase, respectively), PVN (5·75-fold increase) and LH (2·68-fold increase) of BRO rats. We suggest that early weaning has a long-term effect on the expression of NPY as a consequence of developmental plasticity, and the presence of astrogliosis indicates hypothalamic inflammation that is closely related to overweight and hyperleptinaemia observed in our model.

Key words: Lactation: Developmental plasticity: Central obesity: Neuropeptides: Reactive astroglia

It has been shown that the lactation period is a critical stage of development, and that early weaning could permanently affect the progeny due to malnutrition or changes in neural and hormonal status[1–7]. The developing progeny has a large potential to adapt to nutritional or hormonal changes, and the process that leads to long-term alterations has been referred to as 'developmental plasticity' or 'programming'[8,9].

Our group has developed a model of programming based on early weaning, in which milk yield is reduced during the last 3 d of lactation through the inhibition of prolactin with the administration of bromocriptine (BRO), a type 2 dopaminergic receptor agonist. The reduction in maternal milk production causes malnutrition in pups and increases the transfer of leptin through the milk, causing hyperleptinaemia

in pups[10]. These alterations are sufficient to induce several metabolic changes in adulthood as a result of metabolic programming, including overweight, higher visceral fat mass, hypothyroidism, hyperglycaemia, insulin resistance, dyslipidaemia, and increased medullary adrenal function and serum glucocorticoid concentration[1,2,11]. In addition, these animals also exhibit hyperleptinaemia and central leptin resistance[1].

Disturbances in leptin levels caused by altered perinatal nutrition lead to long-term consequences for energy metabolism and body mass in adult life[12]. Our group has shown that leptin administered during the lactation period can programme for overweight in the adult rat[13]. One explanation is that leptin may affect the formation and function of hypothalamic circuitries when administered during the

critical period of lactation when neural plasticity is particularly high[14–17].

In the hypothalamus, leptin binds to its long-form receptor Ob-Rb in the arcuate nucleus (ARC), where it positively regulates anorexigenic neurons that release pro-opiomelanocortin (POMC) and its cleavage product, α-melanocyte-stimulating hormone (α-MSH), as well as cocaine- and amphetamine-regulated transcript (CART), and negatively regulates orexigenic neurons that release neuropeptide Y (NPY) and agouti-related peptide[18,19]. These ARC neurons project their axons to other hypothalamic nuclei such as the paraventricular nucleus (PVN) and the dorsomedial, ventromedial and lateral hypothalamus (LH), as well as to extrahypothalamic areas. In the PVN, this circuitry regulates anorexigenic neurons such as corticotropin-releasing hormone and thyrotropin-releasing hormone (TRH)[19,20] involved in energy expenditure.

We have demonstrated in another model of early weaning that the interruption of lactation 3 d earlier than usual, on postnatal day (P) 18, without the use of pharmacological substances or maternal separation, using a bandage that covers all the mothers' teats, causes malnutrition and hypoleptinaemia in pups; in adulthood, the progeny is programmed for overweight, hyperphagia, higher visceral fat mass, hypertriacylglycerolaemia as well as insulin and leptin resistance[6]. This phenotype was evidenced by alterations in the hypothalamus, such as increased NPY expression and decreased CART expression in the PVN[7]. The two models of early weaning that we have previously studied differ in terms of the following: (1) neonatal leptinaemia (hyperleptinaemia in the pharmacological model and hypoleptinaemia in the non-pharmacological model); (2) hyperphagia (only observed in the non-pharmacological model); (3) thyroid function in adulthood (hypothyroidism in the pharmacological model and euthyroidism in the non-pharmacological model). Therefore, we hypothesised that the changes in the neural circuitry as a result of the programming effect could be different in the two models despite the similar changes in serum leptin levels and effects in adulthood.

To assess the possible differences between the two models, in the present study, we investigated whether early weaning (3 d earlier than usual, at P18) by pharmacological (injections of bromocriptine) inhibition of maternal prolactin induces hypothalamic alterations in adult Wistar offspring. We analysed the expression of neuropeptides NPY, POMC, α-MSH and CART in the following hypothalamic nuclei: ARC; PVN; LH. Furthermore, it has recently been proposed that before the onset of obesity, hypothalamic inflammation with the release of pro-inflammatory cytokines and gliosis can be observed[21–23]. Therefore, we also studied whether in the present model, rats programmed by early weaning exhibited astrogliosis that could be suggestive of hypothalamic inflammation.

Experimental methods

The experimental design was approved by the Animal Care and Use Committee of the Instituto de Biologia Roberto Alcantara Gomes of the Universidade do Estado do Rio de Janeiro (CEUA/048/2010; CEUA/061/2011), and conducted according to the principles established in the Brazilian Law no. 11.794/2008. The experiments followed the ethical doctrine of the three 'R's (reduction, refinement and replacement)[24], minimising the number of animals and the suffering caused by the experimental procedures.

Animals

Wistar rats were maintained in a temperature-controlled vivarium (25 ± 1°C) with an artificial dark–light cycle (lights on 07.00 hours and lights off 19.00 hours). Pregnant rats were placed in individual cages with free access to water and food. To avoid the influence of litter size on programming, only dams with litter sizes of at least ten to twelve pups were used. At birth, litters were adjusted to six male pups per dam to maximise lactation performance. In a few cases where there were no six male pups in the same litter, pups were cross-fostered from other litters that were born on the same day.

Experimental model of programming by maternal prolactin inhibition using bromocriptine

Lactating rats were divided into two groups: BRO group (n 7), which was treated with 0·5 mg bromo-α-ergocryptine (Novartis) intraperitoneally, twice per d (1 mg/d), at the end of the lactation period, from P18 to P21; control group (n 7), which was treated with saline injections using the same protocol described above.

After progenitor separation (at P21), body mass and relative food intake (g/100 g body weight) of three male pups from each group were monitored every 7 d until P180. Throughout the entire experiment, both groups received the same standard Nuvilab® chow, with 1466 kJ/100 g (350·5 kcal/100 g) of solid food (carbohydrate 55 g, protein 22·5 g, fat 4·5 g and 8 g fibre; composition: calcium carbonate; maize bran; soyabean meal; wheat bran; dicalcium phosphate; NaCl; vitamin/mineral premix; amino acids; antioxidants). Food ingestion was calculated as the difference between the weight of the food remaining in the food bin and the amount of food placed 7 d before and that value was divided by 4, which was the number of animals in each cage. To measure serum glucose level, blood was drawn from the tail tip of each animal after 12 h of fasting and assessed using a glucometer (Accu-Chek Advantage; Roche Diagnostics).

BRO and control offspring were killed at P180. Furthermore, one rat offspring per litter, which was randomly chosen from the three rats from the same litter maintained in the same cage, was used for immunohistochemistry.

Antibodies

Anti-NPY (rabbit polyclonal antibody, diluted 1:1000) and anti-glial fibrillary acidic protein (anti-GFAP) (mouse monoclonal antibody, diluted 1:200) were purchased from Sigma-Aldrich. Anti-CART (goat polyclonal antibody, diluted 1:100)

and anti-POMC (rabbit polyclonal antibody, diluted 1:100) were purchased from Santa Cruz Biotechnology, Inc. Anti-α-MSH (sheep polyclonal antibody, diluted 1:10 000) was purchased from EMD Millipore Corporation.

The application of primary antibodies was followed by the application of appropriate secondary antibodies, which were purchased from Molecular Probes (Invitrogen) and used at 1:400 dilution: donkey anti-rabbit conjugated with Alexa Fluor 488; donkey anti-mouse conjugated with Alexa Fluor 555; donkey anti-goat conjugated with Alexa Fluor 555; donkey anti-sheep conjugated with Alexa Fluor 488.

Immunohistochemistry

BRO and control offspring were perfused at P180 (*n* 7 rats per group). The rats were fasted for 12 h. They were anaesthetised with Avertin® (0·3 mg/kg intraperitoneally) and intracardially perfused with a saline solution followed by 4% paraformaldehyde and then by paraformaldehyde plus 10% sucrose. The brain was sectioned at 20 μm using a cryotome (Hyrax C25; Zeiss) and stored at −20°C. All coronal sections containing the hypothalamus starting from the bregma (−1·88 mm), according to Paxinos & Watson[25], were collected on gelatinised slides. For immunohistochemical procedures, the sections were treated with a 0·3% PBS–Triton solution followed by incubation with a blocking solution (5% bovine serum albumin), and then immunolabelling with primary antibodies was performed. Immunoreactivity was visualised by incubation with an appropriate secondary antibody, and the sections were counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (diluted 1:5000; Sigma-Aldrich). The slides were mounted in ProLong Gold Antifade Reagent (Invitrogen, Molecular Probes). In control procedures, omission of primary antibodies with the inclusion of the secondary antibody produced no labelling.

Image capturing was performed using an epifluorescence microscope (Olympus BX-40). For quantification procedures, we used captured images of four coronal sections from each animal. Each selected section was separated from other selected sections by 100 μm. The following hypothalamic nuclei were analysed: medial ARC (mARC) and lateral ARC (lARC); PVN; LH.

Anti-NPY and anti-α-MSH antibodies specifically label neuron fibres, CART labels fibres and cell bodies, and anti-GFAP labels astrocyte processes and cell bodies. For the quantification analysis of immunostaining, we used Image-Pro Plus (version 4.5; Media Cybernetics, Inc.). The segmentation tool was initially used to better differentiate the background from actual anti-NPY, anti-CART, anti-α-MSH or anti-GFAP staining. Because the cut-off point was selected by the experimenter (who was blinded to the group assignments), the segmentation tool procedure was repeated three times on separate occasions for each image. The grey-scale image (brighter pixels indicating stronger labelling) obtained from this procedure was used in the quantification of the average pixel brightness density of the entire image. Then, we calculated the average pixel density that represents the fibre density of the three treated images from each original image, and this final result was used as the datum.

For the quantification of POMC, CART and GFAP, the cells positive for each marker were counted in the captured images (four sections counterstained with DAPI per nucleus per animal) of the selected hypothalamic nuclei. Because individual cells can be easily identified by perinuclear labelling, the number of positive cells associated with each marker was determined.

Statistical analysis

Data are presented as means with their standard errors. Body weight, food intake and glycaemia data were analysed by Student's *t* test. Regarding immunohistochemistry data, to reduce the likelihood of type 1 statistical errors that might result from the repeated testing of the global dataset of each immunostaining procedure, results for the density of NPY, CART, α-MSH and GFAP immunostaining in seven animals from each group (control and BRO) were first evaluated using a global repeated-measures ANOVA. Separate repeated-measures ANOVA were performed for each immunostaining procedure. Group (control or BRO) was considered as the between-subjects factor. Number of nuclei (NPY: ARC, PVN and LH; CART: ARC, PVN and LH; α-MSH: PVN and LH; GFAP: mARC, lARC, PVN and LH) was considered as the within-subjects factor. Data regarding the number of positive cells showing CART and GFAP immunostaining (considering GFAP as a marker of astroglia) in seven animals from each group were also subjected to repeated-measures ANOVA using the same between-subjects factor. Number of nuclei (CART: ARC, PVN and LH; GFAP: mARC, lARC, PVN and LH) was considered the within-subjects factor. Regarding POMC, data regarding the number of positive cells in the lARC were subjected to a univariate ANOVA using group as the between-subjects factor. For simplicity, results based only on the averaged univariate *F* tests are reported. Lower-order ANOVA were used whenever significant effects or interactions were observed in the global repeated-measures ANOVA. For main effects, significance was considered at $P < 0.05$. For significant interactions at $P < 0.1$, we also examined whether lower-order main effects were detectable after the subdivision of interactive factors[26].

Results

In adulthood (P180), body weight was higher in the BRO group (Fig. 1(a)). No difference was observed in food intake during the entire experiment (Fig. 1(b) and (c)). Glycaemia was also higher in the BRO group (Fig. 1(d)). These results confirm the previous data from our group using this model[1,2].

Previously, serum leptin level, fat mass and feed efficiency were evaluated in the other two rats from the same litter used in the present experiment; as we needed to perfuse the rats in the present study, we could not easily obtain the serum and fat tissue. Those results were recently published[27] together with an analysis of bone metabolism. In summary, at P180, the BRO group was programmed for hyperleptinaemia (+97·6%, $P < 0.05$) and exhibited higher feed efficiency
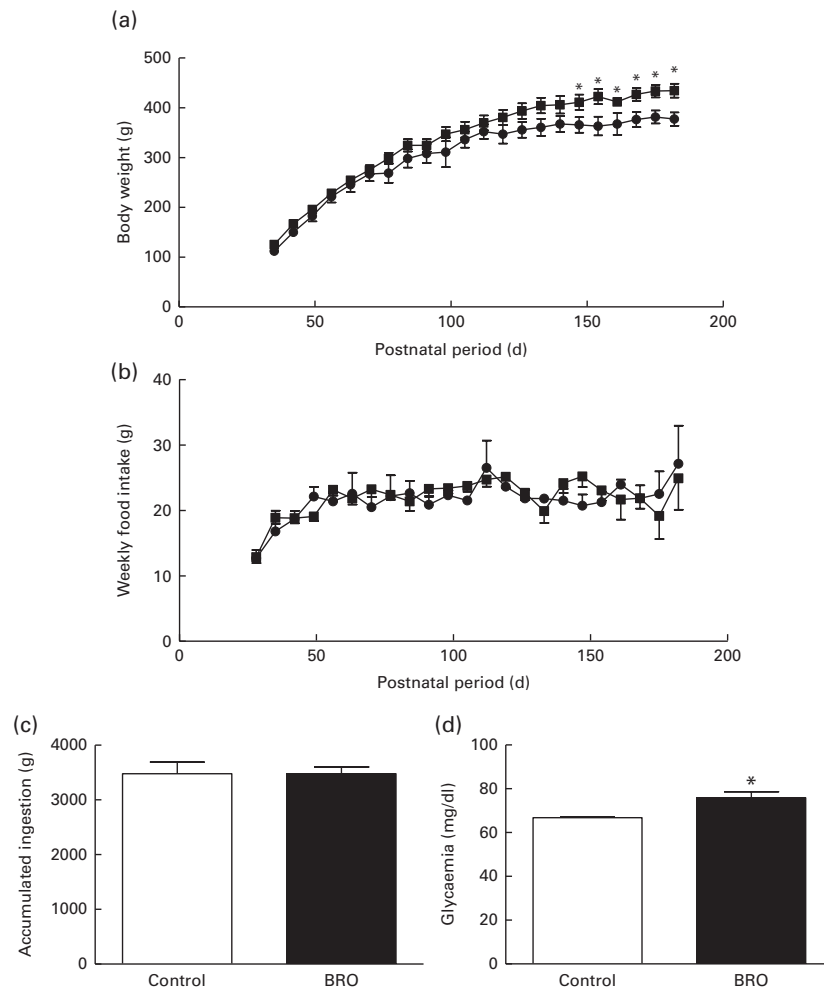
Fig. 1. (a) Body weight and (b) food intake in rats with different age groups, (c) accumulated ingestion and (d) glycaemia at postnatal day (P) 180. After weaning, (a) body weight and (b) food intake were monitored every 7 d until P180. (c) Accumulated ingestion is the sum (g) of food intake per animal throughout the entire period. Although there was an increase in the body weight of bromocriptine (BRO) rats, no differences were found in food intake. (d) Serum glucose was evaluated at P180. Values are means (n 5 rats per group), with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group (P < 0·05; Student's t test). ●, Control; ■, BRO. To convert glycaemia in mg/dl to mmol/l, multiply by 0·0555.

(+10 %, P<0·05) and higher visceral fat mass (retroperitoneal: +85 % and epididymal adipose tissue: +58 %, P<0·05).

The qualitative analysis of NPY immunostaining showed a denser NPY-positive fibre network at P180 in the ARC (Fig. 2(b) and (d)) and PVN (Fig. 2(f) and (h)) of BRO offspring than that of control offspring (Fig. 2(a), (c), (e) and (g)). The quantitative analysis of the intensity of NPY immunostaining showed a significant increase in the ARC (+82 %; F = 10·3, df = 1, P=0·008) and PVN of the BRO group (+83 %; F = 5·7, df = 1, P=0·034). No differences were observed between the groups regarding the intensity of NPY immunostaining in the LH nuclei (F = 1·6, df = 1, P>0·10) (Fig. 3(a)) .

Regarding the anorexigenic neuropeptides, in the lateral part of the ARC (containing POMC/CART neurons), the qualitative and quantitative analysis of CART immunostaining did not reveal any differences between the BRO and control groups in terms of the number of CART-positive cells in the ARC, PVN and LH nuclei (P>0·10; Fig. 3(b)), nor did the analysis of the intensity of CART immunostaining in all nuclei (P>0·10; Fig. 3(c)). POMC immunohistochemistry showed

no qualitative or quantitative differences in the total number of POMC-positive cells between the groups (P>0·10; Fig. 3(d)). The POMC cleavage product, α-MSH, also did not show differences in immunostaining intensity between the groups in the PVN and LH nuclei (P>0·10; Fig. 3(e)).

Astrocytes in the hypothalamus of the control group were mostly distributed around the vessels and around the third ventricle (Fig. 4(a) and (g) and arrows in Fig. 4(b), (h) and (i)). In the BRO group, astrocytes were more evenly distributed throughout the tissue; the number of astrocytes was higher in all nuclei, including the two different regions of the ARC: the medium part (containing most of the NPY/ agouti-related peptide cell bodies) and the lateral part (containing most of the POMC/CART cell bodies). The BRO group showed a 98 and 53 % increase in the mARC and lARC, respectively (mARC: F = 22·0, df = 1, P=0·002; lARC: F = 9·7, df = 1, P=0·014), an 89 % increase in the PVN (F = 16·1, df = 1, P=0·004), and a 54 % increase in the LH nuclei (F = 5·9, df = 1, P=0·04) (Fig. 5(a)). The BRO group presented astrocytes with higher immunoreactivity to GFAP
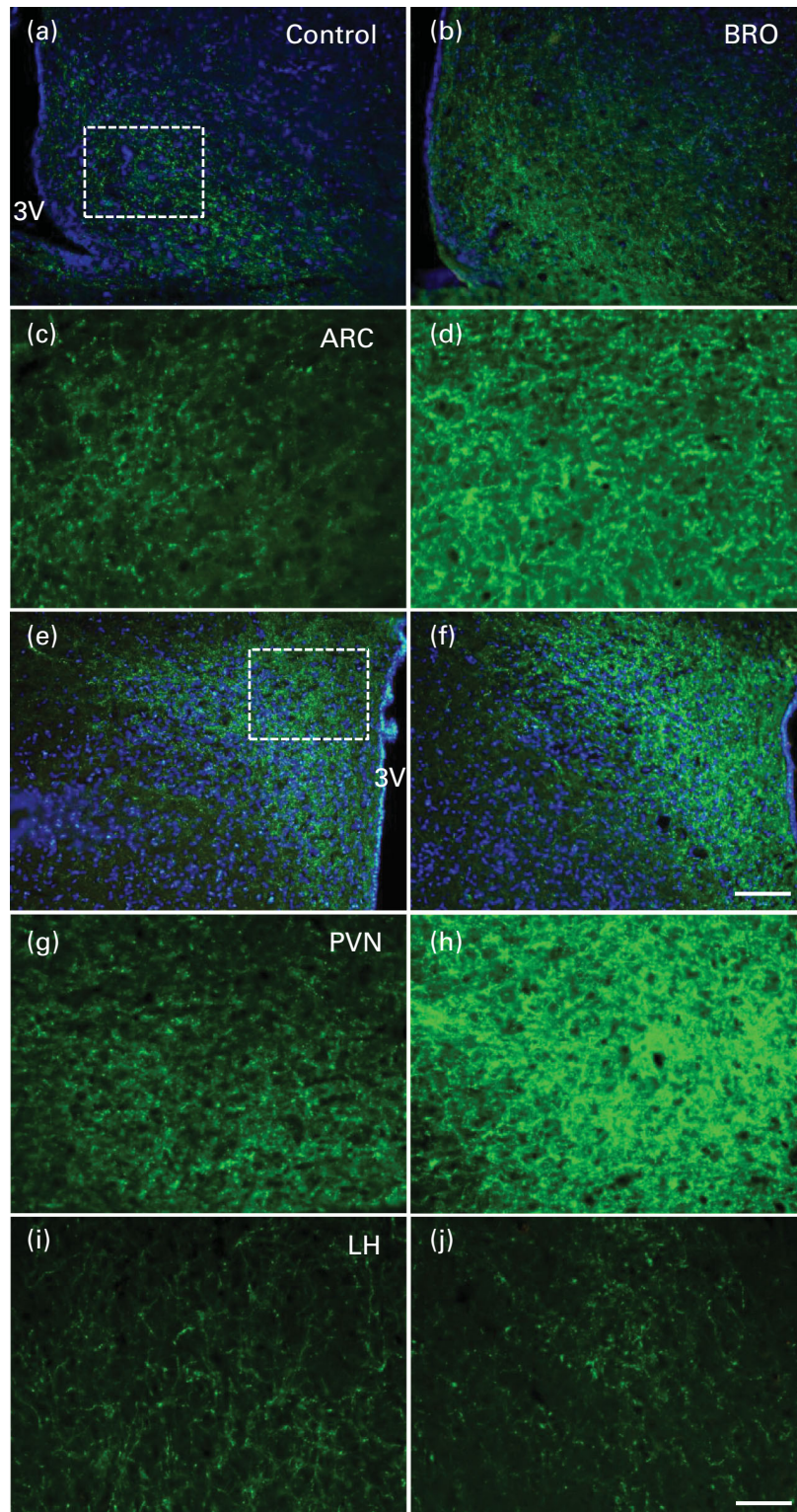
Fig. 2. Immunohistochemistry of neuropeptide Y (NPY) in the arcuate nucleus (ARC), paraventricular nucleus (PVN) and lateral hypothalamus (LH) at postnatal day 180. Immunohistochemistry was carried out after the perfusion of control rats (n 7) and rats programmed by maternal prolactin inhibition using bromocriptine (BRO rats, n 7). The anti-NPY antibody was detected with a secondary antibody conjugated with Alexa Fluor 488 (green), and sections were counterstained with DAPI (blue). (a, b) ARC (lower resolution). The marked area in (a) shows the medial part of the ARC. (c, d) ARC (medial part); (e, f) PVN (lower resolution). The marked area in (e) shows the parvocellular region. (g, h) PVN; (i, j) LH. There was an increase in NPY immunoreactivity in the (d) ARC and (h) PVN of the BRO group. 3V, third ventricle. (a, b, e, f) Scale bar, 100 μm; (c, d, g–j) scale bar, 50 μm. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).
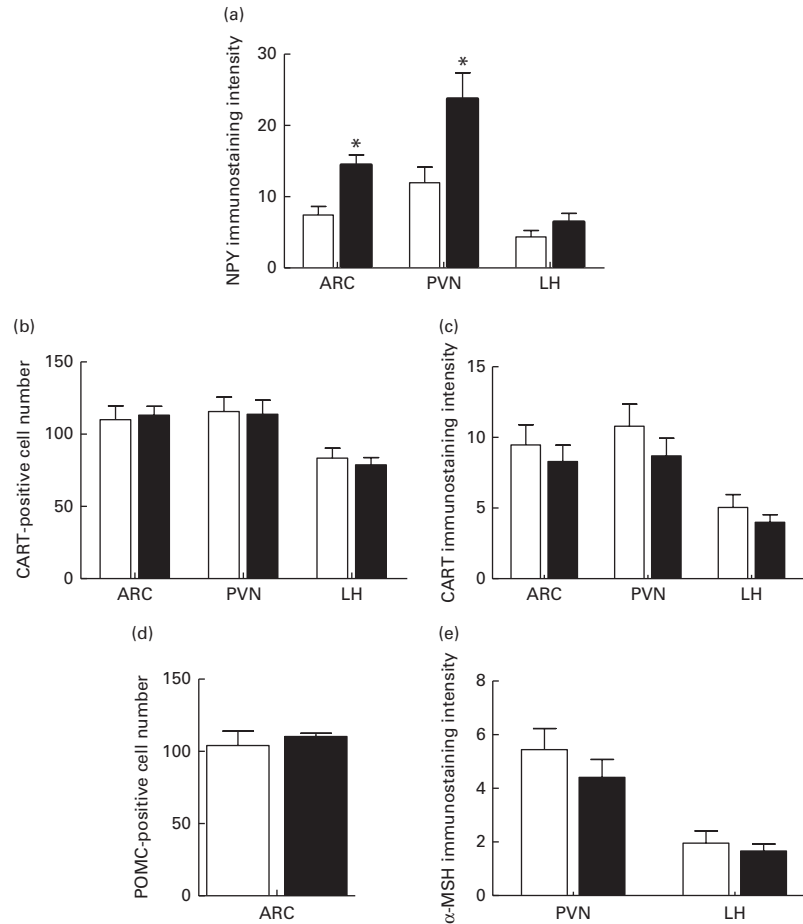
Fig. 3. Quantitative data of immunohistochemistry analysis for the hypothalamic orexigenic peptide neuropeptide Y (NPY) and the anorexigenic peptides cocaine-and amphetamine-regulated transcript (CART), pro-opiomelanocortin (POMC) and α-melanocyte-stimulating hormone (α-MSH) in rats at postnatal day 180. Quantification was performed by photomicrography of different hypothalamic nuclei taken from four coronal sections of each animal. To analyse the neuron fibres of (a) NPY, (c) CART and (e) α-MSH immunostaining, we used the segmentation tool and expressed as average pixel intensity. Quantitative data of (b) CART and (d) POMC immunostaining are expressed as the number of CART/POMC-positive cells. NPY analysis was carried out in the arcuate nucleus (ARC), para-ventricular nucleus (PVN) and lateral hypothalamus (LH). An increase in ARC and PVN immunostaining can be observed in the BRO group. CART analysis was carried out in the ARC, PVN and LH nuclei. POMC cell count was carried out in the ARC, while its cleavage product, α-MSH, was analysed in the PVN and LH nuclei. Values are means (n 7 rats per group for NPY, CART and α-MSH; n 5 rats per group for POMC), with their standard errors represented by vertical bars. □, Control; ■, BRO. * Mean value was significantly different from that of the control group ($P < 0.05$).

and cellular hypertrophy (arrowheads in Fig. 4(f)), with over-lapping processes of different astrocytes, which indicate astrogliosis. The quantitative analysis showed an increase in the intensity of GFAP immunostaining in all the nuclei of the BRO group: 6·06-fold increase and 9·13-fold increase in both mARC and lARC, respectively (mARC: $F = 33·1$, df = 1, $P<0·001$; lARC: $F = 96·9$, df = 1, $P<0·001$); 5·75-fold increase in the PVN ($F = 17·7$, df = 1, $P=0·003$); 2·68-fold increase in the LH ($F = 31·5$, df = 1, $P=0·001$) (Fig. 5(b)).

Discussion

In the present study, we evaluated whether hypothalamic alterations in neuropeptide expression occurred in the adult offspring of dams that underwent prolactin inhibition for 3 d before weaning at P21. We have previously reported an increase in NPY and a decrease in CART immunostaining in the PVN of adult obese rats that were programmed using a non-pharmacological early weaning (NPEW) model, in

which the teats of lactating rats were blocked with a bandage to prevent lactation[7] for the same period as that used for the BRO model in the present study. The BRO group also showed an increase in NPY expression in both PVN and ARC. However, regarding the anorexigenic neuropeptides CART, POMC and α-MSH, no changes were observed.

Our group has a great interest in the study of metabolic pro-gramming as a cause of obesity and other metabolic disorders during adulthood. Different models of hormonal, nutritional or environmental changes during the lactation period in rats have been studied. The model used in the present study has been well studied in adult animals and for the metabolic changes presented (higher body weight, hyperleptinaemia, hypothyroidism, insulin resistance, among others), so we decided that this period is important for a better understand-ing of the central mechanisms of energy regulation. It will be very interesting to evaluate these phenomena in other periods (e.g. adolescence, sexual maturation and senescence); however, this needs to be done in parallel with all of the

phenotypic characterisations. Moreover, it is possible that this model could present sexual dimorphism due to the treatment with bromocriptine in mothers during the lactation period. Sexual differences in other models of metabolic programming, such as maternal nicotine exposure during lactation[28] and maternal flaxseed supplementation during lactation[29], have already been demonstrated. However, whenever females are studied, the hormonal variable can always affect the results, and extra care should be taken to consider the oestrous cycle.

As reported previously, the reduction in maternal milk production by prolactin inhibition causes malnutrition in pups and increases the transfer of leptin through the milk[10]. Interestingly, maternal treatment with bromocriptine did not change the prolactin profile in pups at weaning, but prolactin was lower in adulthood[2]. Adult BRO animals had increased
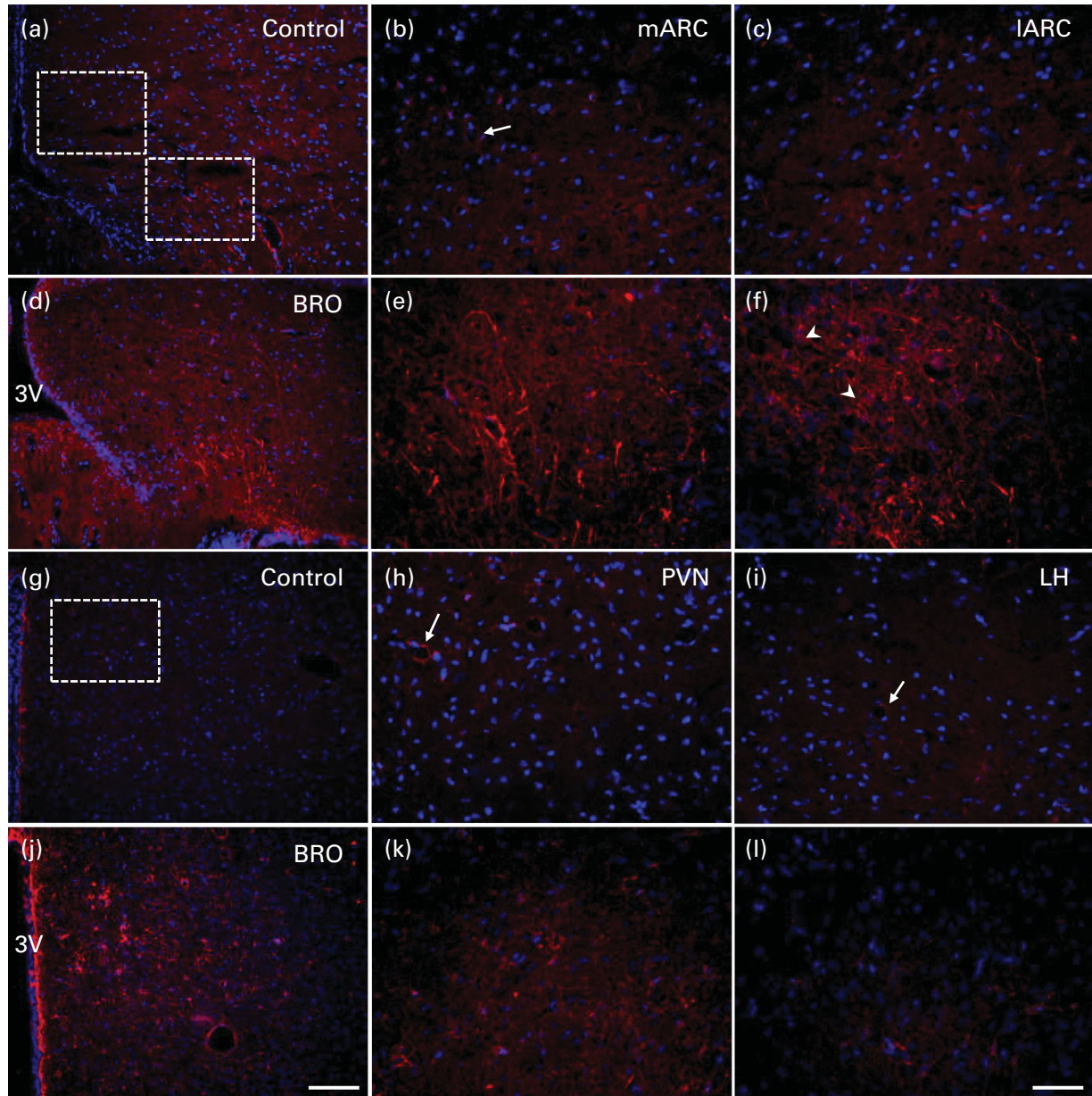

Fig. 4. Hypothalamic astrogliosis in bromocriptine (BRO) rats as revealed by glial fibrillary acidic protein (GFAP) immunohistochemistry in the arcuate nucleus (ARC), paraventricular nucleus (PVN) and lateral hypothalamus (LH) at postnatal day 180. The anti-GFAP antibody was detected with a secondary antibody conjugated with Alexa Fluor 555 (red) and sections were counterstained with DAPI (blue). ARC analysis was carried out in two different regions: medial ARC (mARC, containing most of the NPY/agouti-related peptide cell bodies) and lateral ARC (lARC, containing most of the pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript cell bodies). (a–c, g–i) Control group; (b–d, j–l) BRO group. (a, d) ARC (lower resolution). The marked areas in (a) show the mARC (above) and lARC (below) of the ARC. (b, e) mARC; (c, f) lARC (higher resolution); (g, j) PVN (lower resolution). The marked area in (g) shows the parvocellular part of the PVN. (h, k) PVN; (i, l) LH. The distribution of astrocytes in the control group was preferentially around the vessels (arrows in (b), (h) and (i)). In the BRO group, GFAP immunoreactivity was more intense and astrocyte processes were thicker than those observed in the control group (arrowheads in (f)). 3V, third ventricle. (a, d, g, j) Scale bar, 100 μm; (b, c, e, f, h, i, k, l) scale bar, 50 μm. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).
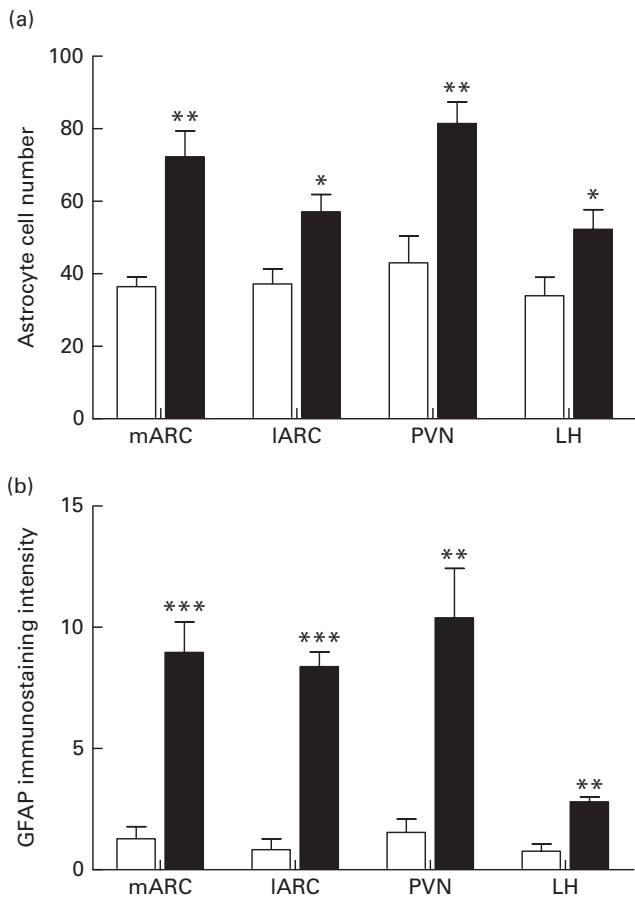
(a)

(b)

Fig. 5. Quantitative data of the (a) number of astrocytes and (b) glial fibrillary acidic protein (GFAP) immunostaining in the arcuate nucleus (ARC), the paraventricular nucleus (PVN) and the lateral hypothalamus (LH) at postnatal day 180. (a) Quantitative data of the number of astrocytes are expressed as the number of GFAP-positive cells. (b) Quantitative data of GFAP immunostaining were analysed using the segmentation tool, and are expressed as the average pixel intensity. ARC analysis was carried out in two different regions: medial ARC (mARC) and lateral ARC (lARC). The number of astrocytes was higher in all the regions of the bromocriptine (BRO) group. This increase in cell number was followed by an increase in the intensity of GFAP immunostaining in all the regions. Values are means (n 5 rats per group), with their standard errors represented by vertical bars. Mean value was significantly different from that of the control group: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. □, Control; ■, BRO.

body mass with central obesity, higher medullary adrenal function, higher corticosteronaemia and insulin resistance[2,29]. However, these animals did not have hyperphagia[1,2,11], as confirmed here. The BRO animals are hypometabolic as they present central hypothyroidism[11]. In addition, they present reduced locomotor activity[30]. BRO animals presented hyperleptinaemia in adulthood, and despite the fact that the total hypothalamic protein content of Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT-3) and suppressor of cytokine signalling 3 (SOCS-3) was not altered, BRO animals presented resistance to the anorectic effects of leptin when injected because food intake was not reduced as observed in the control group[1]. Thus, another leptin-induced signalling pathway, such as the extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, may be altered in this situation.

We have already demonstrated that NPEW rats can exhibit permanent changes in hypothalamic circuitry[7], indicating that the conditions in which the interruption in lactation occurs are crucial for programming outcomes. Hypoleptinaemia is only observed in neonate NPEW rats[6], whereas in nicotine-programmed animals and in the present BRO-programmed model, the weaned pup presents hyperleptinaemia[10,31]. Thus, we suggest that the differences in the neuronal orexigenic/anorexigenic circuitry could be established in this early critical period of developmental plasticity.

Here, we observed an increase in NPY expression in the ARC and PVN of the BRO group, which is a characteristic that is also present in two other models of adult obese and leptin-resistant programmed rats: NPEW rats[7] and rats whose mothers received nicotine during lactation[32]. It is possible that the main cause of the higher NPY expression in the three models is leptin resistance. However, these last two models presented lower activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which is different from BRO programming. Interestingly, the three models also differ in the expression of other anorexigenic neuropeptides, and NPEW rats are the only hyperphagic group and are the only group that exhibit hypoleptinaemia at weaning[6]. It seems that the hyperphagia of NPEW rats can not only be attributed to the higher expression of NPY but also to a decrease in CART expression. Conversely, lower CART expression in NPEW rats is not only caused by leptin resistance because BRO rats showed no change in CART expression. Nicotine-programmed rats are also normophagic but present higher NPY and lower CART expression levels, while compensating for these changes with higher POMC expression[32]. Thus, although similar leptin resistance was observed in the three different models of programmed obesity, the hypothalamic neuropeptides could be differently regulated if different leptin signalling pathways were affected in the hypothalamic nuclei. Thus, it seems that the JAK/STAT pathway is important for the control of CART expression, and lower CART levels are more related to hyperphagia.

It has been previously demonstrated that BRO animals develop central hypothyroidism[11], and one possible explanation is the reduction of TRH. NPY inhibits TRH expression in the PVN, which leads to a decrease in thyroid-stimulating hormone-β mRNA levels in the pituitary and thus lowers T_4 and T_3 production[33]. Therefore, the increase in NPY expression in the PVN could negatively regulate TRH expression in BRO animals. In contrast, it has also been demonstrated that NPY stimulates corticotropin-releasing hormone in the PVN[34]. This is consistent with the hypercorticosteronaemia of BRO animals[2]. We previously observed, in another model of metabolic programming by maternal exposure to nicotine during lactation, the same pattern of increased NPY expression that could be related to lower TRH and higher corticotropin-releasing hormone expression observed in the PVN[32]. This observation is reinforced by the fact that in that nicotine exposure model, the offspring exhibit central hypothyroidism and higher corticosteronaemia in adulthood[28,35].

Leptin concentration is critical to hypothalamic development, which occurs mostly after birth in rats. Projections from the ARC to the PVN and LH during development are completed at P16[14,15,36], just 2 d before lactation interruption in BRO offspring and the onset of the anorexigenic effect of leptin, which only starts after the third postnatal week in rats[14]. Several studies have already shown that malnutrition during critical stages of hypothalamic development can permanently affect the organisation or this brain region. Rocha et al.[37] showed that malnutrition during early life (from P1 to P10) causes a delay in leptin peak during lactation as well as in NPY projection from the ARC to the PVN.

In the present study, we demonstrated significant hypothalamic astrogliosis, which is suggestive of inflammation in various parts of the ARC, PVN and LH nuclei. Some characteristics that define reactive astrogliosis are progressive alterations in molecular expression, progressive cellular hypertrophy, and, in severe cases, proliferation and scar formation[38]. Here, we showed astrocytes with hypertrophy of the cell body and processes, disruption of individual domains (overlapping of astrocyte processes), and an increased number of these cells, which suggest reactive astrogliosis[38]. This is consistent with the idea that hypothalamic inflammation is closely related to obesity[21–23,39]. Indeed, hyperleptinaemia can induce astrogliosis both in vitro and in vivo. It has been suggested that the increase in GFAP expression and alterations in astrocyte morphology can modulate leptin distribution and signalling in neurons[40]. The deletion of leptin receptors in astrocytes alters cell morphology and increases its synaptic inputs on POMC and agouti-related peptide neurons. In addition, the anorexigenic effect of leptin is reduced, indicating an active role of glial cells in feeding behaviour[41].

Obesity also induces astrogliosis and increases the synthesis of inflammatory cytokines, such as IL-6 and PGE$_2$, by astrocytes in the mouse[42], demonstrating the effective role of astrocytes in the development of hypothalamic inflammation. Obese animals programmed by neonatal overfeeding exhibit hypothalamic inflammation based on increased activation of microglia, and this occurs during development and persists until adulthood, demonstrating that glial cells can also exhibit long-term alterations due to developmental plasticity[43]. Our BRO-programmed model produces higher visceral fat mass and hyperleptinaemia, the findings that can be associated with hypothalamic astrogliosis. Because the NPEW model is hyperleptinaemic in adulthood but hypoleptinaemic at P21, it will be interesting to evaluate GFAP expression in the NPEW model to determine whether this gliosis is dependent on leptin concentration in early life or only in adulthood.

In summary, our data provide evidence regarding the relevance of different anorexigenic hypothalamic pathways for the diverse neonatal imprinting effects that are observed in different animal models of early weaning. In contrast, NPY seems to be generally increased in different imprinting models. The relationship between those neuronal circuitry changes and astrogliosis reported here is unknown. However, both changes can be fundamental for the obese phenotype.

Therefore, our data support the hypothesis that a shortened lactation period leads to long-term effects on central neuropeptide expression and gliosis as a consequence of developmental plasticity.

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