Early leptin blockade predisposes fat-fed rats to overweight and modifies hypothalamic microRNAs

Charlotte Benoit^{1,2}, Hassina Ould-Hamouda^{1,2}, Delphine Crepin^{1,2}, Arie Gertler³, Laurence Amar^{1,2} and Mohammed Taouis^{1,2}

¹Neuroendocrinologie Moléculaire de la Prise Alimentaire, University of Paris-Sud, UMR 8195, Orsay F-91405, France

²Neuroendocrinologie Moléculaire de la Prise Alimentaire, CNRS, Centre de Neurosciences Paris-Sud, UMR 8195, Orsay F-91405, France

³Faculty of Agricultural, Food and Environmental Quality Sciences, The Institute of Biochemistry, Food Science, and Nutrition, The Hebrew University of Jerusalem, PO Box 12, 76100 Rehovot, Israel

Correspondence
should be addressed
to M Taouis
Email
mohammed.taouis@u-psud.fr

Abstract

Perinatal leptin impairment has long-term consequences on energy homeostasis leading to body weight gain. The underlying mechanisms are still not clearly established. We aimed to analyze the long-term effects of early leptin blockade. In this study, newborn rats received daily injection of a pegylated rat leptin antagonist (pRLA) or saline from day 2 (d2) to d13 and then body weight gain, insulin/leptin sensitivity, and expression profile of microRNAs (miRNAs) at the hypothalamic level were determined at d28, d90, or d153 (following 1 month of high-fat diet (HFD) challenge). We show that pRLA treatment predisposes rats to overweight and promotes leptin/insulin resistance in both hypothalamus and liver at adulthood. pRLA treatment also modifies the hypothalamic miRNA expression profile at d28 leading to the upregulation of 34 miRNAs and the downregulation of four miRNAs. For quantitative RT-PCR confirmation, we show the upregulation of rno-miR-10a at d28 and rno-miR-200a, rno-miR-409-5p, and rno-miR-125a-3p following HFD challenge. Finally, pRLA treatment modifies the expression of genes involved in energy homeostasis control such as UCPs and AdipoRs. In pRLA rat muscle, *Ucp2/3* and *Adipor1/r2* are upregulated at d90. In liver, pRLA treatment upregulates *Adipor1/r2* following HFD challenge. These genes are known to be involved in insulin resistance and type 2 diabetes. In conclusion, we demonstrate that the impairment of leptin action in early life promotes insulin/leptin resistance and modifies the hypothalamic miRNA expression pattern in adulthood, and finally, this study highlights the potential link between hypothalamic miRNA expression pattern and insulin/leptin responsiveness.

Key Words

- ▶ postnatal leptin
- ▶ overweight
- ▶ leptin resistance
- ▶ insulin resistance
- ▶ microRNA
- ▶ high-fat diet

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Introduction

Leptin acts at the hypothalamic level to inhibit food intake and to increase energy expenditure (Ahima & Flier 2000). Through its long isoform receptor OBRb, leptin stimulates anorexigenic and inhibits orexigenic neurons of the arcuate nucleus (ARC) that project to

the paraventricular nucleus (PVN) to adjust energy homeostasis (Schwartz *et al.* 2000).

In the early postnatal period, leptin plays an independent role from regulating body weight and fat size. In newborn rodents, a peak of leptin appears during the

second week of life, which is not related to the regulation of food intake (Ahima *et al.* 1998, Mistry *et al.* 1999, Proulx *et al.* 2002). This peak has been related to a maternal origin through lactation (Stocker *et al.* 2004, Bautista *et al.* 2009). Other studies mentioned an endogen origin based on positive correlations between leptin plasma levels and leptin adipose tissue mRNA expression during the second week of life (Kirk *et al.* 2009). Furthermore, in newborn rats, leptin plasma levels were correlated with leptin mRNA expression in both cortex and pituitary gland (Morash *et al.* 2001). At this stage, leptin also regulates the expression of hypothalamic neuropeptides (Proulx *et al.* 2002) and contributes to the hypothalamic neural network maturation through the outgrowth of neuronal projections from the ARC to paraventricular, dorsomedial, and lateral hypothalamic nuclei that begins from day 6 after birth in rodents (d6) and ends at the second postnatal week (Bouret *et al.* 2004a,b, Valerio 2006, O'Malley *et al.* 2007). In leptin-deficient *ob/ob* mice, hypothalamic projections are disturbed and partially restored following postnatal leptin treatment (Bouret *et al.* 2004a,b). In addition, newborn rats treated with leptin exhibit hyperleptinemia, hyperinsulinemia associated with a higher body weight gain, and hypothalamic leptin resistance at the adulthood (Toste *et al.* 2006, Vickers *et al.* 2008, Kirk *et al.* 2009). We have recently shown that blocking leptin action using a leptin antagonist predisposed newborn rats to body weight gain and leptin resistance at adulthood (Attig *et al.* 2008). Consequently, all events that affect leptin action during this developmental period such as inappropriate diet of dams during pregnancy (high caloric diet or energy restriction) impair hypothalamic neuronal projections and promote later in life metabolic and endocrine disorders (Kirk *et al.* 2009, Patel *et al.* 2009).

This clearly indicates that the alteration of leptin levels in early life similarly affects energy homeostasis and insulin/leptin responsiveness through most likely different mechanisms but with identical consequences. These mechanisms are not yet elucidated; thus, we hypothesized that high leptin levels impair leptin signaling and this could be mimicked by leptin antagonist. Therefore, leptin antagonist may constitute an important pharmacological tool to further investigate the underlying mechanisms of leptin action at this stage.

As leptin is crucial for hypothalamic neural organization in early life, we hypothesize that postnatal leptin may induce and initiate deep changes at the transcriptional and posttranscriptional levels. Indeed, posttranscriptional events such as RNA stabilization are

important for cell differentiation. MicroRNAs (miRNAs) have emerged as powerful regulators acting at the posttranscriptional level implicated in a wide range of cellular functions. miRNAs are small endogenous RNAs with 20–24 nucleotide length regulating gene expression by base pairing their 2–7 first nucleotides (seed region) with complementary sequences principally located in the 3' UTRs of protein-coding transcripts (Lai 2002). Each miRNA can possibly interact with multiple transcripts and the translation of each transcript can be regulated by multiple miRNAs. Bioinformatics' tools using pair-base analyses estimate that one miRNA can recognize several hundreds of predicted targets (Krek *et al.* 2005). miRNAs have been first known to be implicated in development (Fiore *et al.* 2008) or oncogenesis (Cho 2007). Furthermore, compelling evidence has demonstrated the substantial regulatory role of miRNAs in energy metabolism and liver functions (Poy *et al.* 2004, Esau *et al.* 2006, Plaisance *et al.* 2006). miRNAs are also implicated in neuronal development, dendritic spine formation, synaptic plasticity (Vo *et al.* 2005, Schrott *et al.* 2006, Smalheiser & Lugli 2009), as well as in the regulation of signaling pathways (Inui *et al.* 2010). However, little is known concerning the expression profile of miRNAs in the hypothalamus and whether leptin can modulate it in early life. Thus, we hypothesized that leptin blockade early in life may affect hypothalamic miRNA expression pattern leading to a specific phenotype characterized by the predisposition to increased body weight gain associated with leptin and insulin resistance.

Here, we investigated the impact of pegylated rat leptin antagonist (pRLA) treatment (from d2 to d13) on body weight gain and insulin/leptin resistance at both hypothalamic and peripheral levels, as well as the effect on the expression profile of hypothalamic miRNAs.

Materials and methods

Animals

Twenty one-mated Wistar rats aged 8 weeks were purchased from Janvier (Le Genest-St-Isle, France) at the stage of d15 of gestation. They were housed individually in fixed conditions of temperature (20–22 °C) and hygrometry (around 40%) in a 12 h light:12 h darkness cycle. They had free access to food (standard chow diet) and water. At birth (d0), litters were 11 pups and then were adjusted at ten pups per dams as described previously (Férezou-Viala *et al.* 2007). All male pups were kept in each litter and the adjustment was made by reducing or increasing the number of female pups. Litters were

randomly assigned to the control or treated group. Newborn male rats received a daily s.c. injection of NaCl (0.9%, $n=36$, 5 l, control group) or pRLA (7.5 $\mu\text{g/g}$ per day; $n=35$, 5 l, treated group) between 1700 and 1800 h, from d2 to d13. The dose of pRLA was chosen according to our previous paper (Attig *et al.* 2008). pRLA was a gift from Protein Laboratories Rehovot Ltd. (Rehovot, Israel). pRLA was prepared from RLA (Salomon *et al.* 2006) by monopegylation according to the protocol described by Elinav *et al.* (2009) for leptin antagonists. It is specific for leptin receptor only and binds to leptin receptors from all mammalian species. Though pegylation reduces pRLA's affinity toward leptin receptor by five- to sixfold, its *in vivo* activity is much more potent than the non-pegylated RLA due to its highly prolonged half-life in circulation (A Gertler and G Solomon, unpublished data). After weaning (d28), rats of both groups were fed *ad libitum* with a standard chow diet (377 kcal/100 g; energy derived from carbohydrates, 68.5%; from lipids, 11.9%; and from proteins, 19.6%; formula 113, Safe, Augy, France) and had free access to water. Then, from d125 to d153, all rats were challenged *ad libitum* with an unpalatable high-fat diet (HFD; 439.7 kcal/100 g; energy derived from carbohydrates: 38.5%; from lipids: 46%; and from proteins 15.5%; formula 235 HF, Safe) (Fig. 1A). HFD was replaced every 2 days. All procedures were conducted according to the guidelines of laboratory animal care and were approved by the local governmental commission for animal research: Ethic Committee for animal experimentation of PARIS Center and South# 59 (FRANCE), with authorization # 91-519.

Metabolic and endocrine measures

Body weights were daily measured from d2 to d15 and then once a week. Food intake and body length were measured once a week from weaning (d28) to the end of the experiment. BMI was calculated as the ratio of body weight (g):body length (cm)².

At d28, d90, or d153, fasted (4 h) control and pRLA rats were anesthetized with isoflurane (<5 min in order to avoid any negative impact on measured parameters (Hamaya *et al.* 2000, Takamura *et al.* 2008) before killing. Liver and abdominal adipose tissues were weighted. Blood was collected in heparinized tubes (Lovenox, 200 IU/ml), centrifuged (1500 g during 30 min), and plasma were stored at -20°C . Plasma glucose levels were measured with glycemia kit (Accu-Chek Performa); leptinemia, insulinemia, and adiponectinemia were quantified using ELISA kits (Millipore, Molsheim, France) according to the manufacturer's instructions. Homeostasis model

Figure 1

(A) Experimental design. Rats received a daily i.p. injection of NaCl or pRLA from d2 to d13. Both groups were weaned at d28 with a chow diet and challenged with a HFD from d125 to d153. Rats were killed at d28, d90, or d153 (vertical arrows). (B) Effect of the postnatal leptin blockade on the body weight gain during lactation, chow diet, and after 1-month challenge with HFD. Rats were divided into two groups at birth and daily injected with NaCl (control group, open bars) or leptin antagonist (pRLA group, dark bars). Both groups were weaned at d28 with a chow diet and challenged with a HFD from d125 to d153. Body weights were measured each day during postnatal injections and then once a week until the end of the experiment. Body weight gain after postnatal injections (d2–d28), under chow (d28–d125), and high-fat (d125–d153) diets are presented. The results are expressed as mean $\pm$ s.e.m. $n=35$ per group from d2 to d28, $n=30$ per group from d28 to d90, and $n=15$ per group from d90 to d153.

* $P<0.05$, **** $P<0.0001$ compared with control.

assessment (HOMA) index was calculated following the formula: $\text{HOMA index} = (\text{blood glucose (mg/dl)} \times \text{insulin } (\mu\text{U/ml})) / 405$.

Protein analysis

Liver and hypothalamus of 90-day-old ($n=5$ per group) and 153-day-old ($n=5$ per group) rats were quickly removed and frozen in liquid nitrogen and stored at -80°C . Samples were homogenized in 1 ml lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5% nonidet-P40, 1% Triton X-100, protease inhibitor cocktail (0.35 mg/ml phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, and 2 mg/ml aprotinin), and phosphatase inhibitor cocktail (10 mM sodium

fluoride, 1 mM sodium orthovanadate, 20 mM sodium β -glycerophosphate, and 10 mM benzamidine) with Precellys 24/Cryolys (hypothalamus: 20 s; liver: 2×20 s). Homogenates were incubated for 2 h at 4 °C and then centrifuged (1 h at 14 000 g at 4 °C) and supernatants were stored at -80 °C. Protein concentrations of supernatants were determined using a protein assay kit (BCA Protein Assay Kit, Thermo Scientific, Courtaboeuf, France).

Leptin sensitivity was assessed by the measurement of leptin-dependent phosphorylation of ERK1/2 in the liver at d90. Control and pRLA-treated rats received an i.p. injection of recombinant rat leptin (1 mg/kg; $n=5$ per group) or NaCl 0.9% ($n=5$ per group) 30 min before killing.

Proteins (70 μ g) were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Blots were blocked with 3% BSA and then incubated in the presence of anti-pERK1/2, anti-p-JNK, or anti-PTP1B for liver extracts, whereas hypothalamus blots were incubated with antibodies directed toward PTP1B, SOCS-3, JAK-2, or IRS-2. Membranes were then incubated with the appropriate secondary antibody and targeted proteins were revealed using enhanced chemiluminescence reagents (ECL, Amersham Life Science). The intensity of bands was determined using Carestream apparatus. Relative protein quantities were normalized using anti-tERK1/2, anti-t-JNK, or anti- β -tubulin antibodies.

***In vitro* insulin stimulation on liver membranes**

Liver (500 μ g) from control and pRLA of 153-day-old rats were homogenized in buffer A (0.32 M sucrose, 2 mM HEPES, pH 7.4, and protease and phosphatase inhibitor cocktail) with tissue homogenizer (Precellys 24/Cryolys). Homogenates were then diluted at 10%w/v in buffer A and centrifuged (5 min at 1000 g at 4 °C). Supernatants were kept and pellets were re-suspended in buffer A and centrifuged (5 min at 1000 g at 4 °C). The two supernatants were mixed and centrifuged (20 min at 1000 g at 4 °C). Pellets were then re-suspended in lysis buffer B (2 mM HEPES, pH 7.4, 50 μ M Ca^{2+} , and protease and phosphatase inhibitor cocktail, incubated for 30 min at 4 °C and centrifuged (20 min at 20 000 g at 4 °C). Pellets, representing crude liver membranes, were suspended in buffer C (50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 2 mM EGTA, and protease and phosphatase inhibitor cocktail) and stored at -80 °C. Protein concentrations of the resulting lysates were determined using a protein assay kit (BCA Protein Assay Kit, Thermo Scientific).

Crude liver membranes (50 μ g) were incubated for 10 min at 37 °C with insulin (1 μ M) and ATP (5 mM) or

with buffer C as control. Reaction was stopped by the addition of loading buffer. Western blots were performed as described earlier, using an anti-phosphotyrosine, an anti- β -insulin receptor, and an anti-IRS-1 antibody.

RNA analysis

Skeletal muscle, liver, adipose tissue, and hypothalamus of 28- ($n=4$ per group), 90- ($n=5$ per group), or 153- ($n=5$ per group) day-old rats were quickly removed in RNase-free conditions, immediately frozen into liquid nitrogen, and stored at -80 °C. Frozen samples of muscle, liver, adipose tissue, and hypothalamus were homogenized using tissue homogenizer (Precellys 24/Cryolys) and RNA was extracted using TRIzol LS reagent (Invitrogen) according to the manufacturer's recommendations. RNAs (1 μ g) were reverse transcribed (F-572L M-Mulv, Finnzymes, Fontenay-sous-bois, France) and subjected to real-time PCR (Step-One, Applied Biosystems) using adequate primers and Fast SyberGreen Master Mix (Applied Biosystems). Relative cDNA quantities were calculated from cycle thresholds (C_t) and normalized with the housekeeping gene *S18* (*Rps18*) using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001).

miRNA analysis

Pooled hypothalamic total RNA from control and pRLA 28-day-old rats ($n=4$) was analyzed using Taqman Low Density Array (TLDA, TaqMan Array Rodent miRNA A+B Card Set v2.0, Applied Biosystems). Briefly, RNA (900 ng) was reverse transcribed using random primers and the High Capacity cDNA Archive Kit. Then, the resulting cDNAs were subjected to qPCR according to the manufacturer's recommendations. miRNA expression was calculated according to the geometric mean of the 21 miRNAs with the lowest variation among the control and treated conditions. Only miRNAs presenting a fold change expression of at least 1.5 (>1.5 or <0.66) in pRLA rats compared with control and having a C_t inferior to 32 were selected for further analyses.

To validate miRNAs identified by TLDA methodology, quantitative real-time PCR was performed for selected miRNAs for each animal. Briefly, RNA (5 ng) was reverse transcribed using miRNA-specific RT primers (TaqMan MicroRNA Assay, Applied Biosystems) and reagents from the TaqMan MicroRNA Reverse Transcription Kit according to the manufacturer's instructions. Then, miRNAs were amplified using a specific miRNA TM primer (TaqMan MicroRNA Assay) and TaqMan Universal PCR

Master Mix (Applied Biosystems). Relative expression levels were calculated from cycle thresholds and normalized with the housekeeping gene *U87* (*Snord 87*) using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001).

Hormones, western blot antibodies, and qPCR primers

pRLA is a mutated leptin that has conserved the ability to bind leptin receptors without inducing leptin signaling (pRLA; L39A/D40A/F41A). pRLA and recombinant rat leptin were produced as described previously (Salomon *et al.* 2006, Elinav *et al.* 2009). Human insulin was purchased from Sigma.

Antibodies anti-p-ERK1/2, t-ERK1/2, p-JNK, t-JNK, IR- β , IRS-1, and β -tubulin were purchased from Cell Signaling Technologies (Danvers, MA, USA); the anti-phosphotyrosine 4G10 was purchased from Upstate (Millipore, France); the anti-PTP1B and anti-SOCS-3 were purchased from Santa Cruz Biotechnology. Secondary antibodies (from mouse and rabbit) conjugated to peroxidase were purchased from Sigma-Aldrich.

miRNA primers were purchased from Applied Biosystems and mRNA primers from Sigma-Aldrich. The PCR primer sequences used were as follows: UCP2 forward: 5'-TGGCGGTGGTCGGAGATAC-3', reverse: 5'-GGCAAGG-GAGGTCTGTCTGTC-3'; UCP3 forward: 5'-CCCAAAGG AACGGACCAC-3', reverse: 5'-GGTTCTGTAGGCATCCA-TAGTC-3'; AdipoR1 forward: 5'-GCTGGCCTTTATGCTG CTCG-3', reverse: 5'-TCTAGGCCGTAACGGAATTC-3'; AdipoR2 forward: 5'-CCACAACCTTGCTTCATCTA-3', reverse: 5'-GATACTGAGGGGTGGCAAAC-3'; OBRa forward: 5'-TTTCCAAAAGAGAGCGGACAC-3', reverse: 5'-AGGTTGGTAGATTGGATTTCATC-3'; OBRb forward: 5'-ACCACATACCTCCTCACACTA-3', reverse: 5'-AGCAG TCCAGCCTACACTCTT-3'; IR forward: 5'-TGCCAC-CAATCCTTCCGTTCC-3', reverse: 5'-TCCTCCGCCTG CCTCTCC-3'; PTP1B forward: 5'-GCACAGCATGAGCAG TATGAG-3', reverse: 5'-TCCACCCACCATCCGTTTCC-3'; NPY forward: 5'-ATGCTAGGTAACAAACG-3', reverse: 5'-ATGTAGTGTCGCAGAG-3'; POMC forward: 5'-AGG TTAAGGAGCAGTGACTAAG-3', reverse: 5'-CGTCTATGG AGGTCTGAAGC-3'; IL6 forward: 5'-GTTGCCTTCTTGGG ACTGATGTT-3', reverse: 5'-ACTGGTCTGTTGTGGGTGG TATC-3'; NF- κ B forward: 5'-GCGACAGATGGGCTACA-CAGAGG-3', reverse: 5'-TGGAGGAGGACGAGAGAGG CA-3'; TNF α forward: 5'-CTCATTCCTGCTCGTGGCGG-3', reverse: 5'-CCGCTTGGTGGTTTGTCTACGA-3'; S18 forward: 5'-TCCCCGAGAAGTTTCAGCACAT-3', reverse: 5'-CTTCC-CATCCTTCACGTCCTTC-3'.

Statistical analysis

Statistical analyses were performed using Mann-Whitney *U* test for endocrine parameter data, signaling, and gene expression experiments. A repeated-measures two-way ANOVA was used to test changes in body weight and energy intake over time followed by Bonferroni *post hoc* test. Linear regression between insulinemia and glycemia was calculated with values of each animal. The results are expressed as mean $\pm$ S.E.M. and $P < 0.05$ was considered as statistically significant.

Results

Postnatal treatment with leptin antagonist increases body weight gain

Body weight gain was determined in three stages: from d2 to weaning (d28), from weaning to d125, and finally during the HFD challenge (from d125 to d153). In all stages, pRLA rats exhibited a significantly higher body weight gain with +17.8% ($P < 0.0001$), +7.4% ($P < 0.05$), and +32.8% ($P < 0.05$) increase between d2–d28, d28–d125, and d125–d153 respectively (Fig. 1B).

Metabolic and endocrine parameters

The weekly energy intake (kcal/rat) was similar between pRLA and control groups at weaning but was significantly higher in the pRLA group at d90 ($P < 0.001$) and d153 ($P < 0.05$) (Table 1). The body length and BMI were significantly ($P < 0.05$) higher in pRLA group at d28, but these differences were abolished in adulthood (d90) and after HFD (d153) (Table 1). Abdominal adipose tissue and liver weights were not significantly modified in pRLA rats (data not shown).

Leptinemia, as expected, increased with age in both pRLA and control groups, but no significant differences were found between the two groups (Table 1). By contrast, adiponectin plasma levels were decreased with age in control group ($P < 0.01$ between d28–d90 and d90–d153). However, pRLA rats exhibited lower levels of plasma adiponectin compared with control rats at d28 ($P < 0.05$) and d90 ($P < 0.01$) but not after HFD challenge (d153) (Table 1). Insulinemia was significantly increased in pRLA rats at d90 ($P < 0.05$) with a high HOMA index (Table 1). Finally, glycemia was similar between pRLA and control rats (Table 1). Considering the coefficient of determination R^2 , insulinemia and glycemia were correlated in control

Table 1 Metabolic and endocrine parameters measured in control and pRLA rats at weaning (d28), under chow diet (d90), and after 1-month challenge with HFD (d153)

	At weaning (d28)		Under chow diet (d90)		Under HFD (d153)	
	Control	pRLA	Control	pRLA	Control	pRLA
Metabolic parameters						
<i>n</i>	36	35	16	16	15	14
Body weight (g)	82.4±1.3	88.5±1.1 [†]	451.4±5.7	465.1±8.4	559.2±9.2	603.4±20.2 ^a
WEI (kcal/rat)	331±14	337±12	593±6	663±15 ^a	542±13	604±17 ^a
Body length (cm) (<i>n</i>)	14±0.1 (16)	14.8±0.1 [†] (16)	26.4±0.3 (5)	26.7±0.5 (5)	27.6±0.2 (5)	29±0.5 (4)
BMI (g/cm ²) (<i>n</i>)	0.403±0.005 (16)	0.421±0.005 ^a (20)	0.623±0.008 (5)	0.66±0.025 (5)	0.717±0.016 (5)	0.729±0.015 (4)
Endocrine parameters						
<i>n</i>	5	5	5	5	5	5
Leptinemia (ng/ml)	5.22±0.88	3.63±0.77	12.19±1.06	15.58±2.19	17.21±1.79	23.21±3.89
Adiponectinemia (μg/ml)	34.1±2.2	24.9±3.4 ^a	23.3±0.7 ^b	19.0±1.0 [†]	16.7±1.4 ^b	20.3±2.0
Insulinemia (ng/ml)	0.87±0.13	0.65±0.12	0.33±0.26	2.53±0.39 ^a	0.08±0.03	0.24±0.13
Glycemia (mg/ml)	230±21.3	202±9.4	206±6.5	208.8±3.3	156.2±2.3	149.6±6.8
HOMA index	2.16±0.31	1.61±0.29	3.29±0.65	6.24±0.97 ^a	0.19±0.07	0.59±0.29
LR (<i>R</i> ² / <i>P</i> value) ^c	0.73/0.06	0.28/0.35	0.88/<0.01	0.42/0.23	0.005/0.89	0.16/0.49

WEI, weekly energy intake. [†]*P*<0.01; [‡]*P*<0.0001.^aDifference between control and pRLA rat at one stage.^bDifference between the same group at the three stages d28, d90, and d153.^cLinear regression between insulinemia and glycemia, calculated with values of each animal.

rats at d28 ($R^2=0.73$) and d90 ($R^2=0.88$), whereas no correlation was detected in pRLA rats (d28: $R^2=0.28$ and d90: $R^2=0.42$). Under HFD, correlation was lost in both control ($R^2=0.005$) and pRLA rats ($R^2=0.16$; Table 1).

Impact of postnatal leptin blockade on insulin and leptin signaling pathways

As pRLA treatment significantly affected insulinemia, HOMA index, and body weight gain, we investigated signaling pathways involving both leptin and insulin. Leptin treatment significantly ($P<0.01$) increased liver ERK1/2 phosphorylation in control rats but not in pRLA rats at d90 (Fig. 2A). We also tested the liver insulin sensitivity after HFD (d153) in both pRLA and control rats *in vitro* using crude liver membranes. Insulin clearly induced the protein tyrosine phosphorylation in control but to a lesser extent in pRLA rats. Two of the insulin-induced phosphorylated proteins correspond to IRS-1 and the β -subunit of insulin receptor as evidenced by immunoblots using antibodies directed toward these two proteins (Fig. 2B). Following HFD challenge, liver JNK phosphorylation was increased in pRLA rats compared with controls ($P<0.001$, Fig. 2C), but PTP1B (PTPN1) expression was not significantly modified (Fig. 2D).

To further investigate the insulin and leptin sensitivity, we determined at d90 the expression levels of PTP1B and SOCS3 in the hypothalamus of both groups. PTP1B

($P<0.05$, Fig. 2E) and SOCS3 ($P<0.05$, Fig. 2F) were upregulated in pRLA rats compared with control rats.

Postnatal leptin blockade affects the expression of genes involved in the control of energy homeostasis

To investigate whether leptin blockade in early life affects genes involved in the control of energy homeostasis, the expression of several genes was studied in muscle, liver, adipose tissue, and hypothalamus. In muscle, pRLA treatment significantly increased *Ucp2* ($P<0.05$) and *Ucp3* ($P<0.01$) expression levels at d90 as well as *Adipor1* ($P<0.05$) and *Adipor2* ($P<0.05$; Fig. 3A, B, C, and D). However, following HFD, the expression of *Adipor2* was decreased ($P<0.05$) in the pRLA group compared with control rats (Fig. 3D). In liver, the expression of *Adipor1* and *Adipor2* was significantly ($P<0.05$) increased in pRLA rats compared with control rats following HFD (d153) but remained unchanged at d28 and d90 (Figs 3F and 4E). In adipose tissue, pRLA treatment significantly reduced the expression of insulin receptor ($P<0.05$) at d153 (Fig. 3G).

In the hypothalamus, the expression of both short (OBRa) and long (OBRb) isoforms of leptin receptor, insulin receptor, and *Adipor2* was significantly ($P<0.05$) increased in pRLA rats at d28 but not at d90 or d153 (Fig. 4A, B, C, and E). The expression of PTP1B was significantly ($P<0.05$) increased at d90 in the pRLA group (Fig. 4D).

Figure 2

Postnatal leptin blockade decreases leptin and insulin sensitivity. (A) 90-day-old control and pRLA rats were injected intraperitoneally 30 min before killing with either saline or leptin (1 mg/kg) and liver ERK1/2 phosphorylation was assessed. Leptin efficiency on ERK1/2 phosphorylation related to total-ERK1/2 is expressed as percentage of NaCl-injected group. (B) Phosphotyrosine profiles after stimulation with NaCl or insulin (1 μ M) + ATP (5 mM) of crude liver membranes of 153-day-old rats. Protein quantities

were verified with anti- β -insulin receptor and anti-IRS-1 antibodies.

(C) Relative basal phosphorylation of JNK in the liver of 153-day-old control and pRLA rats. (D) Relative basal expression of PTP1B protein in liver of 153-day-old rats. (E) Relative basal expression of PTP1B protein in hypothalamus of 90-day-old rats. (F) Relative basal expression of SOCS3 protein in the hypothalamus of 90-day-old rats. The results are expressed as mean $\pm$ S.E.M. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with control.

Distinctive miRNA expression patterns in the hypothalamus of pRLA-treated rats

To investigate the impact of leptin blockade on hypothalamic miRNA expression pattern, we have performed at d28 a large-scale expression analysis using TLDA methodology to analyze 524 rat mature miRNAs and miRNA-related sequences in pRLA rats compared with controls. As shown in Table 2, the relative expression of 38 out of 524 miRNAs was modified in the hypothalamus

with a fold change of 1.5 when comparing pRLA to control rats. In particular, 34 miRNAs were upregulated and four miRNAs were downregulated in the hypothalamus of pRLA rats at d28.

We have selected five miRNAs, based on their predictive targets that have a potential metabolic role, for qRT-PCR validation in the hypothalamus of rats at d28. To test whether pRLA has a long-term impact on miRNA expression, we analyzed these miRNAs at d90 and d153.

Figure 3

Impact of the postnatal leptin blockade on mRNA expression in peripheral tissues. At d28, d90, and d153, mRNA expression was measured by quantitative RT-PCR in skeletal muscle (A, B, C, and D), liver (E and F), and peri-renal white adipose tissue (G). Results were normalized to ribosomal 518 mRNA levels. The results are expressed as mean $\pm$ s.e.m. $n \geq 4$ per group, * $P < 0.05$, ** $P < 0.01$ compared with control.

At d28, the expression of miR-10a was significantly ($P < 0.05$, Fig. 5A) increased in pRLA rats compared with controls whereas miR-200a (Fig. 5B), miR-409-5p (Fig. 5C), and miR-125a-3p (Fig. 5D) were not significantly increased. At d90, miR-200a was significantly reduced ($P < 0.05$) in pRLA rats whereas miR-10a, miR-409-5p, and miR-125a-3p were not modified. The downregulation of miR-200a is in good agreement with the upregulation of PTP1B (Fig. 4D), as PTP1B is one of the potential miR-200a targets. Following HFD challenge (d153), miR-200a, miR-409-5p, and miR-125a-3p were significantly ($P < 0.05$) upregulated in pRLA but not miR-10a. The upregulation

of miR-200a and miR-125a-3p is in good agreement with the downregulation of IRS-2 (Fig. 6A) and Jak-2 (Fig. 6B) respectively, known as potential targets according to TargetScan (Calbridge, MA USA) and DNA Intelligent analysis micro-T3 softwares (Athens, Greece).

Discussion

An adverse hormonal and metabolic environment during the early postnatal period has long-term consequences on energy homeostasis control promoting metabolic disorders including insulin resistance and obesity (Toste *et al.* 2006, Attig *et al.* 2008, Kirk *et al.* 2009). In this study, we report an increase in body weight gain associated with insulin resistance and changes in hypothalamic miRNA expression profile as a consequence of an early postnatal leptin blockade.

Leptin antagonist treatment from d2 to d13 durably impaired energy homeostasis as evidenced by higher body weight gain of pRLA rats compared with controls when fed chow diet. This effect was exacerbated following HFD challenge, indicating that postnatal leptin alteration predisposes to excessive body weight gain when animals

Figure 4

Impact of the postnatal leptin blockade on mRNA expression in the hypothalamus. Panels A, B, C, D and E show changes in the expression levels of *ObRa*, *ObRb*, *AdipoR2*, *PTP-1B* and *IR*, respectively. At d28, d90, and d153, mRNA expression was measured by quantitative RT-PCR. Results were normalized to ribosomal 518 mRNA levels. The results are expressed as mean $\pm$ s.e.m. $n \geq 4$ per group, * $P < 0.05$, compared with control.

Table 2 TLDA analysis. Normalized expression of miRNAs upregulated or downregulated in pRLA rats analyzed by TLDA at d28. miRNAs in boldface have been validated by qRT-PCR

	Control	pRLA
Upregulated miRs		
miR-200a	1	2.72
miR-125b-3p	1	2.37
miR-421	1	2.24
miR-493	1	2.22
miR-1	1	2.17
miR-182	1	2.12
miR-10a	1	2.09
miR-125a-3p	1	2.09
miR-20b-5p	1	2.01
miR-28*	1	1.89
miR-30d*	1	1.88
miR-378*	1	1.83
miR-206	1	1.80
miR-503	1	1.72
miR-184	1	1.69
miR-139-3p	1	1.67
miR-22*	1	1.65
miR-152	1	1.64
miR-377	1	1.64
miR-199a-3p	1	1.62
miR-24-2*	1	1.61
miR-448	1	1.59
miR-222	1	1.57
miR-879	1	1.57
miR-29a*	1	1.57
miR-190b	1	1.56
miR-203	1	1.55
miR-489	1	1.55
miR-379*	1	1.55
miR-409-5p	1	1.54
let-7i*	1	1.54
miR-23b	1	1.52
miR-16	1	1.52
miR-30e*	1	1.51
Downregulated miRs		
miR-344-5p	1	0.65
miR-505	1	0.61
miR-207	1	0.60
miR-154	1	0.57

were fed inappropriate diet as we have previously shown for high palatable diet (Attig *et al.* 2008). The blockade of leptin action mimics the absence of leptin in the early postnatal period, which is known to deeply alter hypothalamic neural circuitry organization as reported in leptin-deficient *ob/ob* mice (Bouret *et al.* 2004a,b). This could, at least partially, explain the impairment of body weight and energy homeostasis controls in pRLA rats. Indeed, postnatal leptin blockade has a significant impact on metabolic and endocrine parameters at adulthood. At d90, this treatment led to insulin resistance as evidenced by hyperinsulinemia associated with increased HOMA index and a lack of correlation between glycemia and insulinemia.

In addition, the plasma levels of adiponectin, considered as an antidiabetic hormone (Kadowaki & Yamauchi 2005), was significantly reduced in pRLA rats, re-enforcing then the insulin-resistant state.

The hormonal environment of pRLA rats is clearly in favor of an insulin-resistant state known to promote leptin resistance. Indeed, insulin and leptin share several signaling pathways such as JAK-2/STAT-3, IRS/PI3kinase, and ERK1/2 kinase (Carvalho *et al.* 2001, Benomar *et al.* 2005a,b) and cross-desensitization of leptin and insulin signaling pathways has been demonstrated (Benomar *et al.* 2005a). The phosphotyrosine phosphatase 1B (PTP1B) was identified as a key element in the cross-desensitization between leptin and insulin pathways, as the over-exposure to both hormones led to the upregulation of PTP1B, which in turn dephosphorylates key signaling components such as IRS-1/2 and JAK-2 (Benomar *et al.* 2009, Berthou *et al.* 2011).

Following HFD challenge (d153), pRLA rats exhibited liver insulin resistance as supported by reduced insulin-dependent IR and IRS-1 phosphorylation. In addition, liver JNK phosphorylation in pRLA rats was increased, which is a hallmark of cellular pro-inflammatory state. The phosphorylation of JNK is commonly associated with insulin resistance through the phosphorylation of IRS proteins on serine residues (Aguirre *et al.* 2000). However, following HFD, other pro-inflammatory markers such as

Figure 5

Impact of the postnatal leptin blockade on hypothalamic miRNA expression. Panels A, B, C and D show changes in the expression levels of miR-10a, miR-200a, miR-409-5p and miR-125a-3p, respectively. At d28, d90, and d153, miRNA expression was measured by quantitative RT-PCR. Results were normalized to *U87* levels. The results are expressed as mean $\pm$ s.e.m. $n \geq 4$ per group, * $P < 0.05$ compared with control.

Figure 6

Downregulation of hypothalamic IRS-2 and JAK-2 in pRLA rats following HFD challenge. At d153, IRS-2 (Panel A) and JAK-2 (Panel B) protein expression was measured by western blot using adequate antibodies. The expression level was normalized to β -tubulin. Band density was measured using Carestream apparatus. The results are expressed as mean $\pm$ s.e.m. $n=4$ per group, * $P<0.05$ compared with control.

TNF α , IL6, and NF κ B were not affected in pRLA rats at both hypothalamic and hepatic levels (data not shown). It is noteworthy that the impairment of liver insulin responsiveness did not significantly affect liver weight; this could be attributed to the length of HFD challenge period (28 days). Indeed, it has been reported that hypothalamic inflammation markers appeared after 8 weeks of HFD challenge (Calegari *et al.* 2011). Furthermore, changes in liver weight have been reported in non-alcoholic fatty liver disease subsequently to at least 4 months of HFD challenge (Tsuchiya *et al.* 2013). Beside the alteration of liver insulin signaling in pRLA rats, we demonstrated that the alteration of leptin signaling in liver was determined by the loss of leptin-dependent ERK1/2 phosphorylation at d90.

Interestingly, hypothalamic PTP1B and SOCS3 were upregulated in pRLA rats promoting both leptin and insulin resistance, which is in good agreement with our

previous findings in human neuroblastoma cell line (Benomar *et al.* 2009). Additionally, hypothalamic OBRa, OBRb, and AdipoR2 were upregulated in pRLA rats at d28 probably as a consequence of low levels of leptinemia and adiponectinemia. In addition, insulin receptor was also upregulated at d28 in pRLA rats and this is in good agreement with the downregulation of miR-344-5p (Table 2). Indeed, insulin receptor is a potential target of miR-344-5p according to TargetScan software.

Importantly, early leptin blockade impairs the expression of key genes involved in energy homeostasis control such as UCPs and AdipoRs. Indeed, at d90, muscle *Ucp2* and *Ucp3* genes, involved in the regulation of muscle thermogenesis, were upregulated in pRLA rats. This could explain that pRLA and control rats exhibited similar body weight at d90, despite the higher energy intake of pRLA rats through an increase in energy expenditure. This hypothesis is supported by previous studies reporting the upregulation of *Ucp3* in insulin-resistant fat-fed animals (Samec *et al.* 1999).

In addition, muscle AdipoRs were upregulated in pRLA at d90 and this led to higher energy expenditure probably through the increase in muscle fatty acid β -oxidation as previously reported (Yoon *et al.* 2006). Following HFD challenge, *Adipor1* and *Adipor2* were upregulated in the liver of pRLA rats, and such upregulations have been reported in type 2 diabetes (Beylot *et al.* 2006, Metais *et al.* 2008).

Importantly, our findings brought new insights into the role of leptin in the expression pattern of hypothalamic miRNAs in early life. Postnatal leptin blockade led to the upregulation of 34 miRNAs and the downregulation of four miRNAs in the hypothalamus of d28-old rats. For the quantitative RT-PCR validation, we have focused on four miRNAs: miR-200a, miR-10a, miR-409-5p, and miR-125a-3p that have been strongly linked to metabolic disorders and neuronal differentiation.

In pRLA rats, hypothalamic miR-200a was downregulated at d90 and upregulated following HFD challenge. This downregulation is in good agreement with the upregulation of PTP1B, which is a potential target of miR-200a according to miRNA target prediction software (TargetScan). The upregulation of neuronal PTP1B has been linked to the impairment of both insulin and leptin signaling (Benomar *et al.* 2009). Additionally, metabolic disorders such as diabetes are associated with changes in the expression levels of the miR-200 family at peripheral tissues. Indeed, miR-200a is decreased in the kidney of non-obese diabetic apoE KO mice (Wang *et al.* 2011) and in the liver of spontaneous diabetic non-obese

GK rats (Herrera *et al.* 2010). Interestingly, miR-200a is increased in liver of obese diabetic *ob/ob* mice (Li *et al.* 2009) as well as in liver and pancreatic islets of obese diabetes-resistant B6-ob mice (Zhao *et al.* 2009). These findings indicate that the expression of miR-200a is decreased with diabetes but is increased in cases of obesity or when diabetes is associated with obesity. In our study, the increase in hypothalamic expression of miR-200a at d153 is associated with a higher body weight gain whereas its decrease at d90 is associated with insulin resistance as evidenced by a high HOMA index and hypoadiponectinemia. Furthermore, the upregulation of miR-200a at d153 in pRLA rats is in good agreement with the downregulation of hypothalamic IRS-2, a potential target, re-enforcing then the insulin resistance.

Furthermore, hypothalamic miR-125a-3p was upregulated in pRLA rats challenged with HFD, which may explain the strong downregulation of JAK-2 signaling, one of its potential targets. The JAK-2 signaling pathway is involved in both insulin and leptin signaling. Indeed, previous studies have shown that the expression of the miR-125 family is modulated by diabetes. Indeed, miR-125b is upregulated in vascular smooth muscle of diabetic *db/db* mice (Villeneuve *et al.* 2010) and miR-125a is increased in liver and adipose tissue of diabetic GK rats (Herrera *et al.* 2009). Moreover, an overexpression of miR-125a and miR-125b led to the alteration of insulin signaling pathways by reducing the insulin-dependent Akt and ERK1/2 phosphorylation in SKBR3 cell line (Scott *et al.* 2007).

We also report the upregulation of miR-10a in the hypothalamus of pRLA rats at d28. This miRNA is a potent inducer of neuroblastoma and muscle cell differentiation (Huang *et al.* 2010, Foley *et al.* 2011, Meseguer *et al.* 2011). As leptin is involved in neuronal network organization in the hypothalamus, we suggest that blocking leptin action early in life promotes neuronal differentiation instead of proliferation leading to the impairment of energy homeostasis later in life as our findings suggest.

In addition, following HFD challenge, hypothalamic miR-409-5p was upregulated in pRLA rats. According to miRNA target prediction software (TargetScan), protein kinase inhibitor beta (PKIB) is a potential target of miR-409-5p. PKIB is a member of the cAMP-dependent protein kinase inhibitor family that interacts with the catalytic subunit of cAMP-dependent protein kinase and acts as a competitive inhibitor. Thus, the upregulation of miR-409-5p potentially leads to changes in AMPK activity in the hypothalamus that is involved in AdipoR1/AdipoR2 signaling and consequently affects energy homeostasis. It

is noteworthy that the direct link between miR-409-5p and AMPK activity needs to be validated in cellular or in *in vivo* models.

Taken together, our findings indicate that pRLA treatment modified miRNAs in the hypothalamus and these changes occurred at different stages corroborating the long-term impact of early leptin blockade as altered energy homeostasis control and insulin responsiveness. Furthermore, our study emphasizes the complexity of the mechanisms linking changes in hypothalamic miRNA expression pattern to overall insulin resistance and energy homeostasis regulation. Thus, to deeply dissect this relationship, more intensive studies are needed in this field to potentially link the modulation of hypothalamic miRNAs pattern with the predisposition to overall insulin resistance.

In conclusion, we demonstrate that the blockade of leptin action early in life affects i) leptin and insulin signaling in the hypothalamus and liver; ii) muscle, liver, and hypothalamic expression of key genes involved in energy homeostasis control; and iii) hypothalamic miRNA expression patterns. Our study brings new insights concerning the complexity of leptin action in early life to modulate and adapt hypothalamic response to energy homeostasis control later in life and highlights the potential role of miRNAs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C B and D C performed the experiments and writing of the manuscript; H O-H performed the experiments; A G was involved in the production of leptin antagonist and discussion; L A discussed and participated in the experimental design; M T participated in the experimental design, performed experiments, and wrote the manuscript.

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