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ORIGINAL ARTICLE

Perinatal nutrient restriction induces long-lasting alterations in the circadian expression pattern of genes regulating food intake and energy metabolism

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Objective: Several lines of evidence indicate that nutrient restriction during perinatal development sensitizes the offspring to the development of obesity, insulin resistance and cardiovascular disease in adulthood via the programming of hyperphagia and reduced energy expenditure. Given the link between the circadian clock and energy metabolism, and the resetting action of food on the circadian clock, in this study, we have investigated whether perinatal undernutrition affects the circadian expression rhythms of genes regulating food intake in the hypothalamus and energy metabolism in the liver.
Design: Pregnant Sprague-Dawley rats were fed *ad libitum* either a control (20% protein) or a low-protein (8% protein) diet throughout pregnancy and lactation. At weaning, pups received a standard diet and at 17 and 35 days of age, their daily patterns of gene expression were analyzed by real-time quantitative PCR experiments.
Results: 17-day-old pups exposed to perinatal undernutrition exhibited significant alterations in the circadian expression profile of the transcripts encoding diverse genes regulating food intake, the metabolic enzymes fatty acid synthase and glucokinase as well as the clock genes BMAL1 and Period1. These effects persisted after weaning, were associated with hyperphagia and mirrored the results of the behavioral analysis of feeding. Thus, perinatally undernourished rats exhibited an increased hypothalamic expression of the orexigenic peptides agouti-related protein and neuropeptide Y. Conversely, the mRNA levels of the anorexigenic peptides pro-opiomelanocortin and cocaine and amphetamine-related transcripts were decreased.
Conclusion: These observations indicate that the circadian clock undergoes nutritional programming. The programming of the circadian clock may contribute to the alterations in feeding and energy metabolism associated with malnutrition in early life, which might promote the development of metabolic disorders in adulthood.
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Keywords: metabolic programming; circadian clock; perinatal undernutrition; hypothalamus; food intake

Introduction

Most living organisms exhibit behavioral and physiological rhythms including those associated with sleep wake cycles, blood pressure and liver metabolism. These rhythms are driven by an endogenous timing system called the circadian clock that regulates the expression over a 24-h period of 3–10% of the genes expressed in a given cell.[1–3] The master pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus but similar clock systems capable to generate coordinated circadian outputs of gene expression exist in other hypothalamic nuclei,[4,5] in other brain regions[6–8] and in peripheral tissues such as the liver[9,10] and the intestine.[11–13] The activity of these peripheral clocks is nevertheless under the control of the master SCN clock through neuronal pathways and signals that remains largely to be identified. The molecular basis of the circadian clock is a transcriptional–translational feedback loop consisting of the transcription factors, circadian locomotor output cycles kaput (CLOCK) and brain and muscle arnt-like protein 1 (BMAL1), whose protein products dimerize to induce the

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transcription of Period (Per1–3) and Cryptochrome (Cry1–2) genes, as well as the expression of several transcription factors involved in the regulation of distinct metabolic process including glucose and lipid biosynthesis.[14–16] Accordingly, a wide range of animal and clinical studies clearly indicate that some of the pathological manifestations of the metabolic syndrome arise from a disruption of the circadian clock.[17–21] Moreover, the circadian machinery can be synchronized by nutrient and metabolic signals. Actually, when rats and mice are submitted to a restricted feeding paradigm in which they have free access to food only for 4 h during the daytime, they increase their locomotor activity 2–4 h before the onset of food availability and this anticipatory behavior is associated with phase advances in body temperature, corticosterone secretion, gastrointestinal motility and heart rate.[22,23] These animals also exhibit shifted phases of the circadian expression pattern of clock genes in the liver and other peripheral tissues.[24–26]

The nutritional environment during fetal and early postnatal growth has been shown to have a profound impact on health in later life. Many epidemiological studies have indeed demonstrated that infants born small for gestational age are more prone to develop obesity, hypertension and type 2 diabetes in adulthood.[27–29] Experimental studies have further corroborated that rodents and other animal species submitted to protein or calorie restriction during gestation and/or suckling exhibit hyperphagia,[30–33] insulin resistance,[34,35] reduced leptin sensitivity,[36,37] hepatic steatosis,[38] elevated blood pressure[39,40] and hyperlipidemia.[35,37,38] These observations have been explained by the thrifty phenotype hypothesis, also called metabolic programming or the developmental origins of disease.[41–45] It is hypothesized that perinatal undernutrition sensitizes the offspring to the development of metabolic diseases via epigenetic changes that act during early life to program feeding behavior and energy homeostasis for optimal survival under nutritionally-deficient conditions. The mismatch between the metabolic programming acquired during fetal and neonatal development and the environmental conditions that the individual will confront later in life, would be at the origin of the high susceptibility to develop metabolic disorders. The cellular and molecular mechanisms underlying the programming effects of perinatal undernutrition remain, however, poorly understood.

Given the link between the circadian clock and energy metabolism, and the resetting action of food on the circadian clock, here we sought to determine whether perinatal undernutrition affects the circadian expression rhythms of genes regulating food intake in the hypothalamus and energy metabolism in the liver.

Materials and methods

All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Principles of laboratory animal care (NIH publication no. 85–23, revised 1985), regarding the care and use of animals for experimental procedures. We used male and virgin female Sprague-Dawley rats weighing 200–250 g (Charles River, St Germain Sur L'arbresle, France) at the beginning of the study. On arrival to the laboratory, they were housed either under a 12-h light/dark cycle (lights on at 0700 hours, defined as Zeitgeber time (ZT) 0) or a 12-h reversed light/dark cycle (lights off at 0700 hours, defined as ZT 12) with food and water *ad libitum*. Animals were kept undisturbed for 1 week following which they were mated. After confirmation of mating by the visualization of spermatozoa in a vaginal smear, pregnant rats within each light/dark cycle were housed individually and assigned to a control (C) or a low-protein (LP) group. The C group was fed a control diet containing 20% of protein (200 g protein kg^{-1}) whereas the LP group was fed an isocaloric low-protein diet containing 8% of protein (80 g protein kg^{-1}).The diets were purchased from Ab Diets (Woerden, The Netherlands) and their composition have been described previously.[35,46] Dams were fed *ad libitum* and their body weight and daily food intake documented every 4 days during gestation and lactation. The morning on which new born litters were found was designated as P0 and the litter size was adjusted to eight pups per dam maintaining as close to a 1:1, male : female ratio as possible. At P21, all pups were weaned on standard laboratory chow. Experiments were performed on P17 and P35 male pups in order to evaluate the consequences of maternal protein restriction on both the establishment and the log-term functioning of the circadian clock. Actually, at P17 the circadian clock is not yet fully developed whereas at P35 it has attained a mature state.[47,48] At the end, a total of 10 litters of each control and low-protein restricted dams were studied. The influence of litter was accounted for by using, for each experimental point, pups selected randomly from at least three different litters.

Quantitative RT-PCR experiments

Animals were killed at 4-h intervals from ZT 0 to ZT 24 under *ad libitum* feeding conditions using a rising concentration of CO_2 and cervical dislocation. Quantitative real-time PCR experiments were then conducted on RNAs extracted from whole hypothalamus or liver. Total RNA was extracted using the Trizol reagent (Invitrogen, Cergy Pontoise, France), treated with DNAse (RNAse free) for 30 min at 37 °C and subsequently purified using the mini-column purification kit NucleoSpin from Macherey-Nagel (Hoerdt, France) according to the manufacturer's instructions. The quantity and quality of the purified RNA was evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Illkirch, France). Afterwards, 1.5 μg of purified RNA was reversed-transcribed using Superscript II RNAseH- Reverse-Transcriptase (Invitrogen) in a total volume of 20 μl and the resulting cDNA was diluted 40-fold in DNAse- and RNAse-free water. Thereafter, 5 μl of each cDNA diluted sample

Table 1 Sequences of primers used for the real-time RT-PCR analysis

Gene	*Forward primer*	*Reverse primer*	*Gene bank*
NPY	GTGGACTGACCCTCGCTCTATC	ATGAGATTGATGTAGTGTCGCAGA	M20373
AgRP	CGTGCTACTGCCGCTCTTC	CCCTGCCTTTGCCCAACATC	NM_033650.1
CART	GCTCAAGAGTAAACGCATTCC	AAGAATTGCAAGAAGTTCCTCG	NM_017110
POMC	GAGGTTAAGGAGCAGTGACTAAGA	GTAGCAGAATCTCGGCATCTTCC	NM_033650
CLOCK	TTCGATCACAGCCCAACTCC	ACCTCCGCTGTGTCATCTTCTC	NM_021856
Per1	TTCGGAGCAGGCAAGTGT	GGCAGGCGAGATGGTGTAGTA	AY903230
BMAL1	CAATGCGATGTCCCGGAAGTTAGA	TCCCTCGGTCACATCCCTGAGAAT	AB012600
PGC1α	GCCACTACAGACACCGCACAC	ATTCGTCCCTCTTGAGCCTTTCG	AY237127
FAS	CGCCGTGGTGCTGGAGATTG	CTTGCCGAGGTTGGTGAGGAAG	M_84761
GCK	TGGAGCAGAAGGGAACAACATC	GCGGTCTTCATAGTAGCAGGAG	M_25807
β-Actin	CTATCGGCAATGAGCGGTTCC	GCACTGTGTTGGCATAGAGGTC	EF156276.1

Abbreviations: *BMAL1*, brain and muscle arnt-like protein 1; *CLOCK*, circadian locomotor output cycles kaput; *FAS*, fatty acid synthase; *GCK*, glucokinase.

was used as template for PCR amplification using SYBR Green (Biorad, Marnes la Coquette, France) as fluorogenic intercalating dye and the iCycler iQ Real-Time PCR detection system instrument from BioRad Laboratories (Hercules, CA, USA). PCR parameters were: an initial denaturation step of 5 min at 95 °C followed by 45 cycles of 30 s at 95 °C and 30 s at 60 °C. The primers used for the amplification are presented in Table 1.

Measurement of food intake

Pups aged 28 days were housed individually in metabolic cages (Charles River). Presented in powder form, standard laboratory chow (SAFE, Augy, France), was available *ad libitum* from a hopper recessed in to the front wall of the cage eliminating fouling of the food with urine or feces. Access to food was restricted to a horizontal slot in the hopper that allowed the animal to eat but not remove the food. A hollow in the front portion of the hopper retained any food spilled out from the hopper. Water was dispensed from a bottle fixed to the front wall. After a habituation period of 7 days, during which the animals attained a stable pattern of feeding, food intake was monitored every 4 h for the next 3 consecutive days.

Data analysis

Experimental results are expressed as means ± s.e.m. with n = four–six pups per time point born to at least three different dams. The relative expression levels of the mRNAs in the different hypothalamic samples were calculated using the comparative ΔC_T method[49] and β-actin RNA as housekeeping gene. The applicability of the C_T method was first validated by determining how the amplification efficiencies of the different transcripts including β-actin varied with template dilution. These experiments showed that the efficiency of the PCR amplification was the same for all the genes and that the expression of β-actin was not influenced either by age or the mother diet.[46]

To test for the presence of circadian rhythms, time series data were first analyzed by one-way ANOVA. The existence of a significant effect of time associated with the clustering of the maximum and minimum values for food ingestion or gene expression into two separate time intervals were used as an evidence of the presence of a circadian rhythm. We then performed cosinor analysis to determine the effects of the maternal diet on the circadian parameters of the rhythm for each one of the genes. For this purpose, mRNA expression levels were expressed as ratios to mean expression of the respective gene at ZT 0 and analyzed using the time series analysis seriel cosinor program (http://www.euroestech.com/). Cosinor analysis adjusts by least square approximation raw data to a cosine wave and provides three parameters describing the rhythm: mesor (mean level around which the cosine function oscillates); amplitude (the distance from the mesor to the extremes, peak or nadir, of the oscillation) and acrophase (the time at which the peak of a rhythm occurs in relation to a defined reference time point). The mesor, amplitude and acrophase for each animal and for each gene were obtained and averaged to determine the statistical difference between C and LP pups by Student's *t*-test. To ascertain the differences in the levels of gene expression between the groups at the different time points of the light/dark cycle, data were analyzed by two-way ANOVA with mother diet (control, low-protein) and ZT as factors. When significant effects were found, Bonferroni's test for multiple comparisons was performed. ANOVA and cosinor analysis were performed using the data derived from both the normal and reversed light/dark cycles. The statistical difference between C and LP animals in body weight was evaluated by Student's *t*-test whereas the average in food ingestion at night and during the day was assessed by one-way ANOVA. Statistical significance was set at $P<0.05$.

Results

Effects of protein restriction on body weight and food intake of the dams

Control and low-protein dams exhibited the same weight and ingested the same amount of food during the gestation period (body weight at day 1 of gestation C = 269 ± 14 g vs LP 276 ± 7 g; body weight at day 19 of gestation C = 396 ± 20 g vs

LP 360 ± 10 g; mean daily caloric intake C = 81.45 ± 2.45 kcal 100 g^{-1} of body weight vs LP 85.52 ± 3.83 kcal 100 g^{-1} of body weight). However, in agreement with previous studies,[50–52] alterations in food consumption were observed during lactation. Specifically, 9 days after delivery, rats fed the 8% protein diet reduced in absolute terms their daily food intake by 48% in relation to control dams with a significant diet × time interaction ($F_{11, 154} = 20.95$, $P < 0.0001$) and by 30% when food intake was normalized to body weight (C = 212.57 ± 9.97 kcal 100 g^{-1} of body weight, $n = 7$ vs LP 149.72 ± 12.62 kcal 100 g^{-1} of body weight, $n = 9$, $P < 0.001$). Consequently, the body weight of LP dams was reduced by 25% in relation to that of their control counterparts (C = 335 ± 11 g vs LP 251 ± 9 g, $n = 10$, $P < 0.0001$). The hypophagia in LP dams persisted throughout lactation.

Effects of protein restriction on suckling pups

Body weight. Maternal protein restriction had no effect on litter size or pup survival (mean number of pups per dam C = 12.30 ± 0.45 vs LP 13.00 ± 0.45). In contrast, at birth, the offspring born to LP dams exhibited lower body weight in relation to their control counterparts. Actually, the mean body weight of control litters 1 day after birth ranged between 6.34 ± 0.16 g and 8.63 ± 0.19 g and that of LP litters between 4.86 ± 0.06 g and 6.60 ± 0.09 g such that statistically significant differences between the two groups could be assessed by comparing either the mean body weight of the litters (C = 6.88 ± 0.27 g vs LP 5.87 ± 0.19 g, $n = 10$, $P < 0.01$), or the individual body weight of all the pups (C = 6.85 ± 0.11 g, $n = 55$ vs LP = 5.99 ± 0.09 g, $n = 70$, $P < 0.0001$). This difference in body weight persisted until the end of the experiment. After weaning, however, offspring born to low-protein nourished dams gained weight much faster in relation to their initial body weight than pups of control rats. Thus, whereas the body weight of control animals increased 164% between 21 and 35 days (from 52.46 ± 1.16 g to 138.75 ± 1.6 g), the weight of the undernourished animals during the same period increased by 311%. (from 21.22 ± 0.35 g to 87.32 ± 2.37 g).

Circadian profile of gene expression in the hypothalamus of control and LP pups. As illustrated in Figure 1 and Table 2, the expression of NPY and CART in the hypothalamus of control animals exhibited a daily rhythm with a peak of expression in both cases during the light cycle (NPY F-value = 3.02, $P < 0.05$; CART F-value = 5.60, $P < 0.001$). One-way ANOVA and cosinor analysis of the data also revealed the existence of a rhythm in the case of the expression of POMC with a significant effect of time (F-value = 3.29, $P < 0.05$). NPY also displayed a rhythmic profile of expression by one-way ANOVA in LP pups, but the peak of expression in this latter case was shifted to the left in relation to control animals such that a significant difference in the expression levels of NPY between the two groups could be observed at ZT 4 (mother diet $F_{1, 79} = 7.10$, $P < 0.01$; $ZT_{6, 79} = 2.90$, $P < 0.05$; mother diet × ZT interaction $F_{6, 79} = 3.36$, $P < 0.01$). In contrast, protein restriction abolished the circadian expression rhythm of CART and POMC (Figure 1). In addition, protein-restricted pups showed significant differences in the expression of POMC

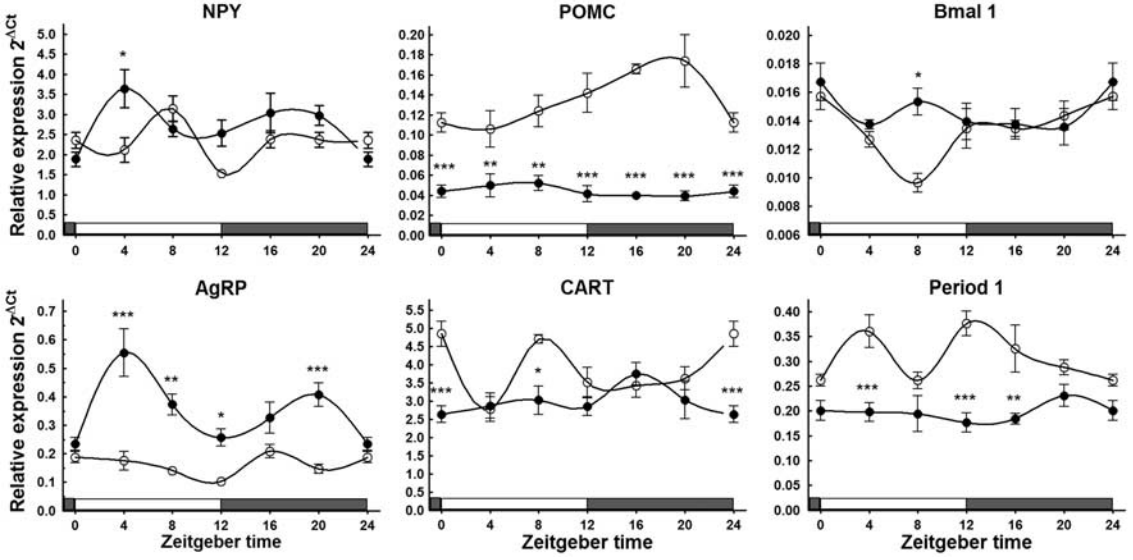

Figure 1 Daily expression profile of orexigenic (*NPY*, *AgRP*) and anorexigenic (*POMC*, *CART*) genes and the core circadian clock transcripts *BMAL1* and *Per1* in the hypothalamus of 17-day-old pups. The hypothalamus was dissected every 4 h from pups born to dams fed a control (open circles) or a low-protein (closed circles) diet during gestation and lactation, and the levels of the transcripts determined by real-time PCR. Values illustrate the relative abundance of each transcript in relation to those of the endogenous β-actin amplified within the same sample and under the same experimental conditions. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (two-way ANOVA followed by Bonferroni's multiple comparison test). Each point corresponds to the mean ± s.e.m. expression levels of four–six pups born to at least three different dams.

Table 2 Circadian rhythm characteristics of gene expression in 17-day-old rats born to dams fed a control or a low-protein diet.

Gene	*Experimental group*	*Percent rhythm*	*Mesor*	*Amplitude*	*Acrophase (h)*	*Shift in peak expression (h)*[a]
Hypothalamus						
NPY	Control	74.49 ± 10.52	92.62 ± 8.09	48.20 ± 3.70	3.43 ± 0.69	
	LP	42.59 ± 4.35	143 ± 6.23***	57.62 ± 9.90	10.52 ± 2.10*	−7.09
AgRP	Control	57.52 ± 14.11	77.79 ± 5.04	32.32 ± 4.69	6.04 ± 1.39	
	LP	62.10 ± 6.93	139.80 ± 15.50**	79.98 ± 12.29**	6.08 ± 0.60	−0.04
POMC	Control	68.87 ± 11.06	121.30 ± 5.66	34.39 ± 7.41	15.72 ± 1.09	
	LP	69.02 ± 12.17	146.00 ± 15.49	22.05 ± 3.19	6.01 ± 1.25***	+9.71
CART	Control	55.76 ± 13.64	74.43 ± 5.79	27.30 ± 2.35	23.05 ± 1.98	
	LP	59.19 ± 10.94	128.00 ± 7.20***	31.58 ± 4.49	12.11 ± 2.62*	+10.94
Bmal1	Control	75.21 ± 12.25	85.33 ± 6.27	15.44 ± 0.82	23.46 ± 2.39	
	LP	83.58 ± 10.49	84.80 ± 3.53	13.22 ± 2.21	12.00 ± 2.61**	+11.46
Period1	Control	52.95 ± 9.67	124.10 ± 5.41	38.64 ± 4.09	11.76 ± 1.15	
	LP	81.12 ± 7.85*	106.80 ± 7.39	45.88 ± 4.77	18.48 ± 1.93**	−6.72
Liver						
FAS	Control	84.06 ± 3.19	318.00 ± 42.42	366.50 ± 32.00	16.60 ± 0.75	
	LP	84.39 ± 9.09	89.27 ± 12.21***	25.59 ± 6.95***	8.63 ± 1.65***	+7.97
GCK	Control	71.18 ± 10.97	606.30 ± 94.79	626.80 ± 135.30	15.10 ± 0.67	
	LP	62.04 ± 7.15	108.70 ± 17.49 **	131.60 ± 27.14**	17.29 ± 1.58	−2.19
PGC1α	Control	55.77 ± 9.66	104.40 ± 4.14	34.74 ± 6.84	15.18 ± 1.11	
	LP	87.63 ± 6.18*	69.24 ± 7.24**	58.56 ± 15.03	21.32 ± 0.95**	−6.14
CLOCK	Control	51.81 ± 11.42	104.30 ± 13.26	64.64 ± 7.96	16.18 ± 1.35	
	LP	68.76 ± 11.13	94.11 ± 10.24	57.45 ± 9.02	21.02 ± 0.54**	−4.84
Period1	Control	61.04 ± 17.52	110.00 ± 7.57	53.03 ± 13.12	5.80 ± 0.95	
	LP	64.16 ± 14.36	125.50 ± 13.91	103.50 ± 24.60	2.75 ± 0.84*	+3.05
Bmal1	Control	85.54 ± 5.38	71.35 ± 5.77	59.27 ± 7.32	21.55 ± 0.23	
	LP	68.95 ± 8.68	46.55 ± 6.37*	54.07 ± 4.24	20.39 ± 1.24	+1.16

Abbreviations: *BMAL1*, brain and muscle arnt-like protein 1; *CLOCK*, circadian locomotor output cycles kaput; *FAS*, fatty acid synthase; *GCK*, glucokinase; LP, low protein. [a]Refers to the advancement (+) or delay (−) in the acrophase of the gene in LP rats in relation to control animals. Single cosinor analysis was performed to obtain the mesor, amplitude and acrophase for each animal and for each gene assuming a 24-h period. Data correspond to the mean of each experimental group. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with control animals (Student's *t*-test, $n=$ four–six animals per group).

and AgRP in relation to controls through the entire circadian cycle (Figure 1). Cosinor analysis of the data confirmed that maternal protein restriction alters the circadian rhythmicity of *NPY*, *AgRP* and *CART* gene expression in the offspring as indicated by the significant differences in mesor and acrophase of the circadian rhythm of these genes in LP pups in relation to control animals (Table 2).

To determine whether protein restriction affects the core circadian clock, we analyzed the rhythmic expression of the mRNA transcripts encoding *BMAL1* and *Per1*. These experiments showed that the expression of these genes follows a circadian pattern in control pups (Per1 F-value = 3.81; BMAL1 F-value = 3.80, Figure 1). In contrast, no circadian rythmicity for Per1 and BMAL1 could be detected in the 17-day-old offspring of dams fed a low-protein diet. The two-way ANOVA analysis of the data further revealed significant differences in BMAL1 and period1 mRNA levels between control and LP pups. Thus, whereas the expression of Per1 and BMAL1 in LP rats was higher than that of control animals at ZT 8, the expression of Per1 at ZTs 4, 12 and 16 in LP rats was significantly reduced in relation to controls (mother diet $F_{1,78}=66.48$, $P<0.0001$; mother diet × ZT $F_{6,78}=2.91$, $P<0.05$).

Circadian profile of gene expression in the liver of control and LP pups. We further explored the impact of protein restriction on the levels of the metabolic enzymes fatty acid synthase (FAS) and glucokinase (GCK) in the liver, as well as on the diurnal expression of the transcripts encoding *BMAL1*, *CLOCK*, *Per1* and *PGC1α*, a transcription factor involved in the reciprocal interaction between the circadian clock and cellular metabolism. As expected, all these genes exhibited a rhythmic pattern of expression in control animals as determined both by cosinor and one-way ANOVA analysis (FAS F-value = 8.28, $P<0.0001$; GCK F-value = 8.38, $P<0.0001$; PGC1α F-value = 2.48, $P<0.05$; CLOCK F-value = 2.44, $P<0.05$; Per1 F-value = 3.01, $P<0.05$; BMAL1 F-value = 2.59, $P<0.05$ Figure 2). Although the rhythmic expression pattern of *PGC1α* and of the clock core genes was preserved in the liver of LP pups (*PGC1α* F-value = 5.40, $P<0.001$; *CLOCK* F-value = 8.97, $P<0.0001$; *Per1* F-value = 3.84, $P<0.01$; *BMAL1* F-value = 4.14, $P<0.01$; Figure 2, Table 2), significant differences in acrophase for all the genes were found between the two groups (Table 2). In contrast, neither FAS nor GCK showed cyclic expression in LP pups. In addition, the offspring of protein-restricted dams showed a significant increase in the expression levels of CLOCK,

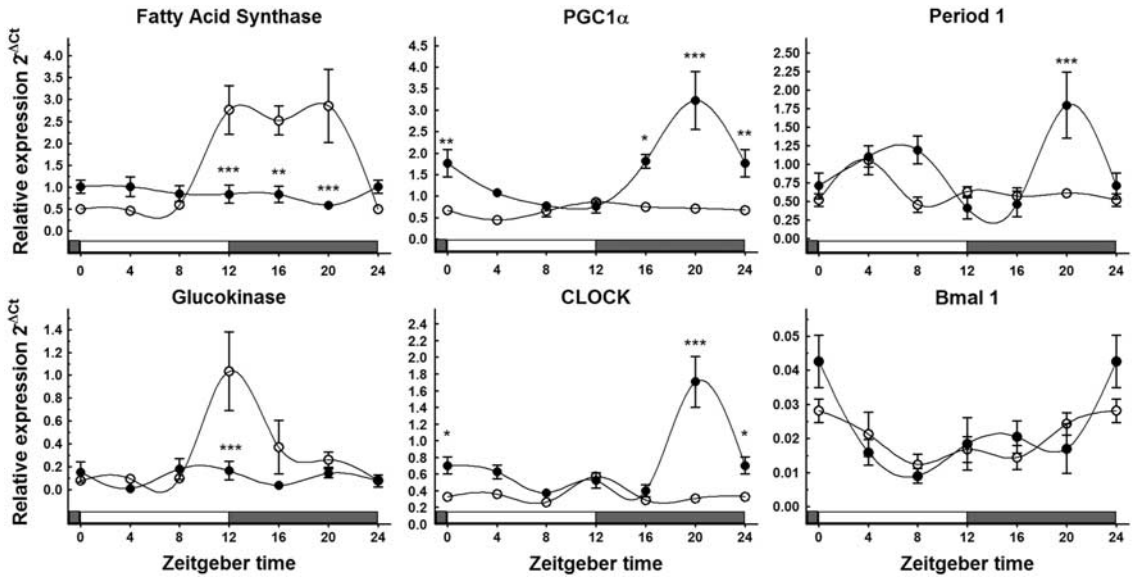

Figure 2 Daily expression profile of FAS, GCK and of the core circadian clock genes in the liver of 17-day-old pups born to dams fed a control (open circles) or a low-protein (closed circles) diet during pregnancy and lactation. The liver was harvested at 4-h intervals over a 24-h period and the levels of the transcripts determined by real-time PCR. Values illustrate the relative abundance of each transcript in relation to those of the endogenous β-actin amplified within the same sample and under the same experimental conditions. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (two-way ANOVA followed by Bonferroni's multiple comparison test). Each point corresponds to the mean ± s.e.m. expression levels of four–six pups born to at least three different dams.

Per1 and PGC1α in relation to controls at different ZTs and these changes were associated with reduced transcription levels of FAS and GCK (Figure 2).

Long-lasting effects of protein restriction

Effects of perinatal protein restriction on the circadian pattern of gene expression in the hypothalamus. At 35 days of age, LP rats still exhibited significant alterations in the circadian expression profile of diverse genes regulating food intake (Figure 3 and Table 3). In particular, the diurnal rhythms of expression of NPY, AgRP and CART in the hypothalamus were abolished in perinatal-undernourished rats and there was a reduction in the maximal levels of AgRP mRNA at ZT 0, 20 and 24 in LP rats in relation to controls (mother diet $F_{1,55}=2.66$; ZT $F_{6,55}=3.35$, $P<0.01$; mother diet x ZT $F_{6,55}=4.99$, $P<0.001$) as well as an increase in the levels of transcripts encoding NPY at ZTs 8 and 12 (mother diet $F_{1,56}=12.61$, $P<0.001$; ZT $F_{6,56}=8.36$, $P<0.0001$; mother diet × ZT $F_{6,56}=4.64$, $P<0.001$).

Consistent with the observations in suckling pups, there was a rhythmic pattern of Period1 mRNA expression both in control and LP 35-day-old rats that was associated with significant reductions in the levels of expression of Per1 in LP rats in relation to controls at ZT 20 (mother diet $F_{1,58}=9.15$, $P<0.01$; $ZT_{6,58}=10.73$, $P<0.0001$, Figure 3) and a shift in the acrophase from 15:40 to 13:04 (Table 3). Similarly, although the diurnal expression pattern of BMAL1 was not altered by the maternal diet (Figure 3), cosinor analysis of the data revealed a significant difference in the acrophase in LP pups in relation to controls (Table 3).

Effects of perinatal protein restriction on the circadian pattern of gene expression in liver. Cosinor and one-way ANOVA analysis showed that the rhythmic expression of FAS in the liver, but not that of GCK, was preserved in 35-day-old LP rats. However, LP rats exhibited a shift in the acrophase associated with significant differences in the expression levels of both genes at ZT 8 in relation to control animals (Figure 4 and Table 3). In contrast, neither the rhythmic expression nor the mRNA levels of the different core genes of the circadian clock in the liver of 35-day-old animals were affected by protein restriction during early life.

Effects of perinatal protein restriction on food intake. In order to determine the long-lasting consequences of protein restriction during perinatal development on the circadian clock at the behavioral level, the daily patterns of food intake were analyzed in 35-day-old control and LP animals fed standard chow since weaning. Both control and LP pups exhibited a typical circadian profile of feeding characterized by the consumption of 74% of their daily food intake during the night period (Figures 5a and b). The offspring of dams fed a low-protein diet consumed, however, more food than control rats during both the light and the dark cycle (diurnal food intake: $C=3.81\pm0.18\,g\,100\,g^{-1}$ of body weight vs $LP=4.97\pm0.23\,g\,100\,g^{-1}$ of body weight, $P<0.001$; nocturnal food intake: $C=10.81\pm0.19\,g\,100\,g^{-1}$ of body

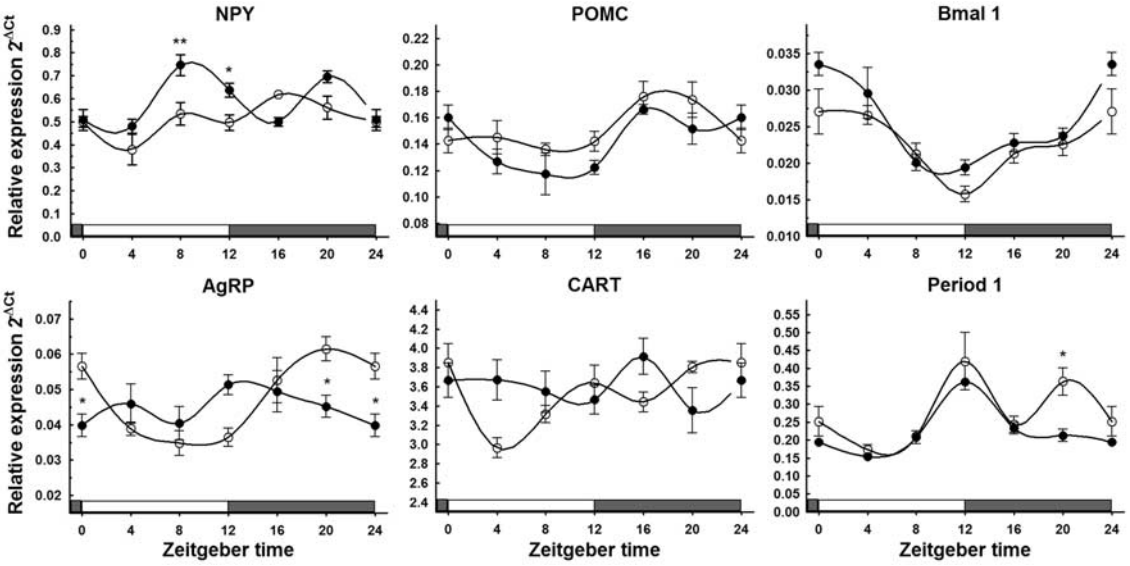

Figure 3 Long-lasting effects of perinatal protein restriction on the daily expression profile of orexigenic (*NPY*, *AgRP*) and anorexigenic (*POMC*, *CART*) genes and the core circadian clock transcript BMAL1 and Per1. Real-time PCR experiments were carried out using cDNA reversed transcribed from total hypothalamus RNA of 35-day-old rats born to dams fed a control (open circles) or a low-protein (closed circles) diet during gestation and lactation and weaned on standard chow at 21 days. Each point corresponds to the relative abundance (mean ± s.e.m.) of each transcript in relation to those of the endogenous β-actin amplified within the same sample and under the same experimental conditions. *$P<0.05$; **$P<0.01$ (two-way ANOVA followed by Bonferroni's multiple comparison test) with four–six different mRNA samples derived from pups born to at least three different dams.

Table 3 Circadian rhythm characteristics of gene expression in 35-day-old rats born to dams fed a control or a low-protein diet

Gene	*Experimental group*	*Percent rhythm*	*Mesor*	*Amplitude*	*Acrophase (hh)*	*Shift in peak expression (hh)*[a]
Hypothalamus						
NPY	Control	74.55 ± 11.73	98.54 ± 2.48	21.73 ± 4.03	15.74 ± 0.85	
	LP	77.47 ± 7.98	118.60 ± 7.53*	38.06 ± 2.57*	7.72 ± 2.16**	+8.02
AgRP	Control	84.18 ± 6.30	78.53 ± 3.38	36.06 ± 0.30	20.94 ± 0.70	
	LP	63.32 ± 11.50	116.70 ± 2.44***	36.20 ± 3.84	14.45 ± 0.54***	+6.49
POMC	Control	67.97 ± 7.40	104.50 ± 4.95	21.75 ± 2.99	15.98 ± 1.01	
	LP	79.96 ± 9.24	88.93 ± 3.94*	19.00 ± 2.49	19.77 ± 0.83*	−3.79
CART	Control	91.46 ± 3.46	91.57 ± 2.48	22.84 ± 2.48	19.59 ± 1.06	
	LP	55.43 ± 12.21*	98.66 ± 2.11	6.76 ± 0.95***	9.44 ± 2.03***	+10.15
Bmal1	Control	90.93 ± 3.10	96.97 ± 5.58	27.20 ± 3.71	4.08 ± 1.15	
	LP	83.30 ± 7.76	71.17 ± 2.72**	20.27 ± 3.20	22.57 ± 0.74***	−18.47
Period1	Control	68.72 ± 11.67	116.00 ± 9.84	41.62 ± 7.62	16.87 ± 1.15	
	LP	76.11 ± 6.62	121.90 ± 9.86	58.00 ± 8.55	15.35 ± 0.92	+1.52
Liver						
FAS	Control	82.77 ± 6.93	79.79 ± 3.92	35.47 ± 4.99	18.44 ± 0.50	
	LP	95.05 ± 1.59	73.64 ± 2.07	55.81 ± 2.88**	20.09 ± 0.27*	−1.65
GK	Control	89.69 ± 6.43	109.00 ± 12.79	85.95 ± 2.69	19.92 ± 0.94	
	LP	78.49 ± 12.86	71.96 ± 7.07	82.23 ± 5.67	4.69 ± 1.16***	+15.23
PGC1α	Control	82.14 ± 6.26	236.40 ± 10.97	131.90 ± 14.70	11.42 ± 0.62	
	LP	92.28 ± 4.34	163.90 ± 13.55**	55.66 ± 9.67**	10.04 ± 1.14	+1.38
CLOCK	Control	83.98 ± 5.03	92.41 ± 10.07	37.35 ± 5.09	5.07 ± 0.73	
	LP	95.32 ± 2.18	94.86 ± 7.89	44.82 ± 6.94	5.13 ± 0.66	−0.06
Period1	Control	76.27 ± 7.70	1013.00 ± 245	1468.00 ± 151.60	14.48 ± 0.51	
	LP	83.41 ± 7.62	433.90 ± 100*	472.30 ± 98.34***	13.99 ± 0.52	+0.49
Bmal1	Control	94.28 ± 2.99	48.83 ± 3.49	70.37 ± 9.62	2.24 ± 0.73	
	LP	95.85 ± 2.08	51.83 ± 5.02	55.53 ± 5.42	1.85 ± 0.53	+0.39

Abbreviations: *BMAL1*, brain and muscle arnt-like protein 1; *CLOCK*, circadian locomotor output cycles kaput; *FAS*, fatty acid synthase; *GCK*, glucokinase; LP, low protein. [a]Refers to the advancement (+) or delay (−) in the acrophase of the gene in LP rats in relation to control animals. Single cosinor analysis was performed to obtain the mesor, amplitude and acrophase for each animal and for each gene assuming a 24-h period. Data correspond to the mean of each experimental group. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with control animals (Student's *t*-test, $n=$ four–six animals per group).

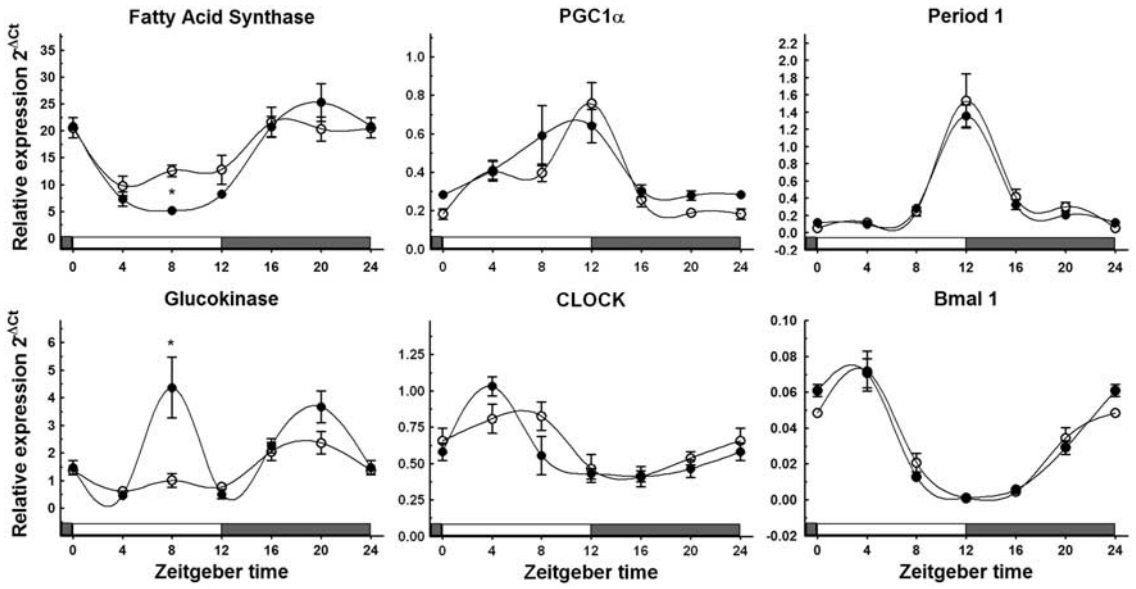

Figure 4 Daily expression profile of FAS, GCK and of the core circadian clock genes in the liver of 35-day-old pups born to dams fed a control (open circles) or a low-protein (closed circles) diet during pregnancy and lactation. The liver was harvested at 4-h intervals over a 24-h period and the levels of the transcripts determined by real-time PCR. Values illustrate the relative abundance (mean ± s.e.m.) of each transcript in relation to those of the endogenous β-actin amplified within the same sample and under the same experimental conditions with four–six different mRNA samples derived from pups born to at least three different dams. *$P<0.05$ in relation to controls (two-way ANOVA followed by Bonferroni's multiple comparison test).

weight vs LP = 14.06 ± 0.44 g 100 g^{-1} of body weight $P<0.0001$, one-way ANOVA, F-value = 321.6, $P<0.0001$). An inspection of the nocturnal pattern of feeding showed the existence of two peaks of feeding in both groups of animals. The first one took place within the 4 h following the extinction of the lights (ZT 16) whereas the second one occurred at the end of the dark photoperiod. During these periods, the levels of food ingestion in perinatally protein-restricted rats were significantly higher than those of controls (Figure 5a). Maternal protein restriction also induced long-lasting changes on the circadian rhythm characteristics of food intake as indicated by the increase in amplitude (from 1.85 to 2.82), and the advance in acrophase (from 19:16 to 18:48), in LP rats.

Discussion

In the present study, we found that feeding a low-protein diet during gestation and lactation induces a long-lasting disruption of the diurnal expression pattern of several genes involved in the regulation of food intake and energy metabolism. This observation strongly suggests that the circadian clock undergoes metabolic programming.

Although it has been repeatedly reported that feeding cues can alter the rythmicity of circadian clocks,[53,54] the herein presented results differ in three main respects from previous observations. First, in contrast to the restricted feeding paradigms used in all the previous studies in which food is provided everyday at the same time only during light-rest periods, here the dams and their offspring had *ad libitum* access to food. Consequently, the modifications of the circadian rhythms, we observed, were not imposed by the temporal consumption of food, but were due to the low-protein content of the diet. Second, in most cases restricted feeding induces a shift in the circadian rythmicity of gene expression without affecting the mRNA levels of the transcripts. In contrast, both a phase change and a reduction in the levels of gene expression were observed in the offspring of dams fed a low-protein diet. Third, and most importantly, although the entrainment of the circadian clock by restricted feeding lasts for a maximum of 2 days after returning to a standard feeding regimen, the effects of perinatal protein restriction on the circadian clock were still present 2 weeks after the low-protein diet was substituted by standard laboratory chow. The hypothesis of the developmental origins of health and disease postulates that the nutritional environment during perinatal development induces long-lasting changes in the physiology and metabolism of the developing organism therefore programming the onset of metabolic and cardiovascular diseases in later life.[42,44,45] We suggest that the fact that protein restriction during gestation and lactation permanently altered the rhythmicity and/or the acrophase of several transcripts in the hypothalamus and liver constitutes *bona fide* evidence that the circadian clock has been nutritionally programmed. This proposition is further sustained by our recent results showing that the resulting offspring of Sprague-Dawley rats submitted to the same metabolic programming model as the one used in this study, exhibit at 6 months, enhanced mRNA hypothalamic levels of PGC1α and CLOCK and of the nuclear receptors RORα and RXRα in relation to control

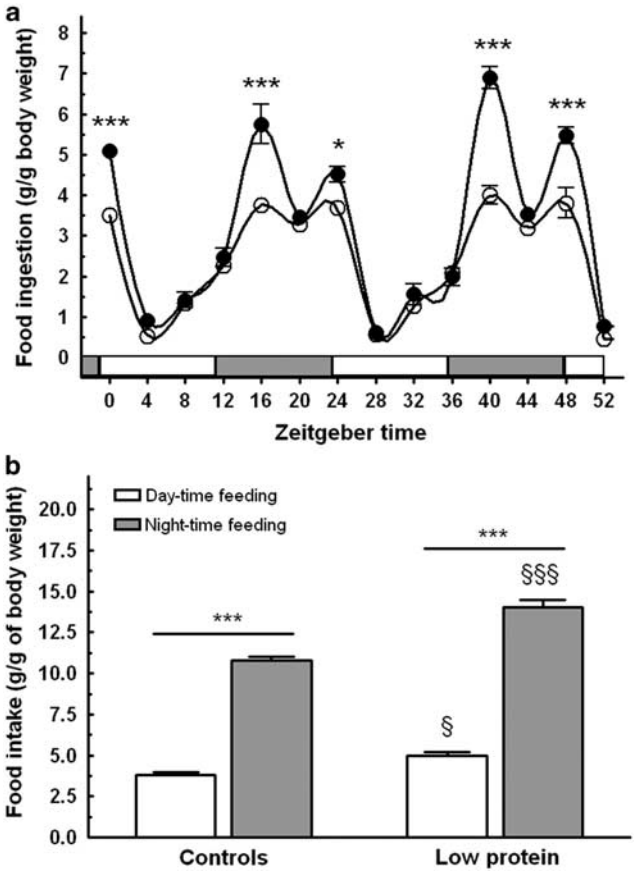

Figure 5 Circadian (**a**) and (**b**) cumulative diurnal and nocturnal food intake of 35-day-old offspring born to dams fed a control or a low-protein diet during gestation and lactation. Data correspond to the mean food intake per 4-h time intervals over a 52-h sampling period measured at 35–38 days of age. The number of animals within each group ranged from 8 to 10. (**a**) ***$P<0.001$, *$P<0.05$ (two-way ANOVA for repeated measures followed by Bonferroni's multiple comparison test). (**b**) $^{§}P<0.05$, $^{§§§}P<0.001$ compared with diurnal and nocturnal, respectively, food intake of control animals (one-way ANOVA). Each point corresponds to the mean ± s.e.m. food intake of four–six pups born to at least three different dams.

animals of the same age.[55] PGC1α and RORα act synergistically to activate BMAL1 expression.[56,57] A direct interaction of RXRα with CLOCK leading to the blockade of the transcriptional activation mediated by CLOCK/BMAL1 has also been demonstrated using a two-hybrid approach.[58]

Interestingly, the changes in the expression of orexigenic and anorexigenic peptides at 35 days induced by early protein restriction mirrored the results of the behavioral analysis of feeding. Thus, in agreement with their increased food intake, LP rats exhibited an increased hypothalamic expression of the orexigenic peptides AgRP and NPY. Conversely, the mRNA levels of the anorexigenic peptides POMC and CART were decreased.

The circadian clock in mammals is organized hierarchically with the clock localized in the SCN of the hypothalamus acting as a central master that synchronizes and entrains the oscillations of the peripheral clocks including those situated in other hypothalamic nuclei.[1,2] Under restricted feeding conditions, however, the rhythms of the peripheral oscillators are reset so that the phases of many physiological functions which are normally under the control of the master clock are aligned with the period of food availability whereas the activity of the SCN clock remains entrained by the light/dark cycle.[22–25] Yet, the rythmicity of the circadian clock in the SCN is also disrupted if in addition to limiting the access to food the amount of calories is reduced to 60–70% in relation to animals fed *ad libitum*.[59–61] We observed that perinatal protein restriction altered in the long term, the circadian rhythmic characteristics of *FAS*, *GCK* and the clock core genes *CLOCK*, *Bmal1* and *Per1* in the liver indicating that the rythmicity of this peripheral clock is disrupted by early undernutrition. We also observed alterations in the circadian expression profile and/or the peak expression levels of NPY, AgRP, CART, Bmal1 and Per1 in the hypothalamus. It was unfortunate that at the moment of dissection the SCN was not separated from the other hypothalamic nuclei. The question therefore remains as to whether the changes in the rythmicity and levels of gene expression in this brain region are the reflection of an alteration of the master clock or are the consequence of an alteration of the oscillators located in other hypothalamic nuclei. Considering that the neurons constituting the SCN represent a very low proportion of the total number of cells in the hypothalamus,[62] we believe that perinatal protein restriction is affecting mainly, if not exclusively, the hypothalamic circadian clocks located outside the SCN. At this moment, however, we cannot formally exclude the possibility that the SCN clock is also affected by perinatal undernutrition. Additional experiments in our laboratory are underway to clarify this point.

The herein presented results indicating that the circadian clock undergoes metabolic programming, add a new view to the field of the developmental origins of disease that might be of special concern for neonatal nutrition and the medical care of IUGR term-born and preterm infants. It is generally acknowledged that preterm infants lack circadian rhythms, but the mechanisms establishing the link between preterm birth and the absence of circadian rhythmicity are not understood.[63,64] Babies born prematurely complete their development in an environment devoid of maternal cues in which their daily light/dark cycles and feeding rhythms are aligned to the working schedule of the medical staff. In adulthood, both light and food signals act as potent Zeitgebergs, but at birth the circadian clock is refractory to light entrainment such that only feeding and other environmental cues, that is, parental care, impinge on its rhythmicity.[65–67] In fact it is not until 3 months after birth that the SCN of the newborn is entirely responsive to the environmental light/dark cycle. Notwithstanding this documented phenomenon, a regular day-night cycle into the Neonatal Intensive Care Unit and intermediate nursery has been introduced as regular practice to improve the circadian synchronization of preterm and term-born infants.[63] Yet, to

the best of our knowledge, the influence of the nutritional status of the preterm infant and/or feeding schedules on the entrainment of circadian rhythms in early life has not been studied to date. Because of the programming effects of perinatal undernutrition on the circadian clock, it will be important in future studies to consider whether the metabolic, sleep and mood disorders that have been associated with a disrupted rhythmicity of the circadian clock result from the combined influence of perinatal undernutrition and postnatal environmental conditions.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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