Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: an epigenetic model of obesity and the metabolic syndrome

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Pre- and neonatal overfeeding programmes a permanent obesity disposition and accompanying diabetic and cardiovascular disorders, by unknown mechanisms. We proposed that early overfeeding may alter DNA methylation patterns of hypothalamic promoter regions of genes critically involved in the lifelong regulation of food intake and body weight. We induced neonatal overfeeding by rearing Wistar rats in small litters (SL) and thereafter mapped the DNA methylation status of CpG dinucleotides of gene promoters from hypothalamic tissue, using bisulfite sequencing. Neonatal overfeeding led to rapid early weight gain, resulting in a metabolic syndrome phenotype, i.e. obesity, hyperleptinaemia, hyperglycaemia, hyperinsulinaemia, and an increased insulin/glucose ratio. Accompanying, without group difference to controls, the promoter of the main orexigenic neurohormone, neuropeptide Y, was methylated at low levels (i.e. < 5%). In contrast, in SL rats the hypothalamic gene promoter of the main anorexigenic neurohormone, proopiomelanocortin (POMC), showed hypermethylation ($P < 0.05$) of CpG dinucleotides within the two Sp1-related binding sequences (Sp1, NF-κB) which are essential for the mediation of leptin and insulin effects on POMC expression. Consequently, POMC expression lacked upregulation, despite hyperleptinaemia and hyperinsulinaemia. Accordingly, the extent of DNA methylation within Sp1-related binding sequences was inversely correlated to the quotients of POMC expression/leptin ($P = 0.02$) and POMC expression/insulin ($P < 0.001$), indicating functionality of acquired epigenomic alterations. These data for the first time demonstrate a nutritionally acquired alteration of the methylation pattern and, consequently, the regulatory 'set point' of a gene promoter that is critical for body weight regulation. Our findings reveal overfeeding as an epigenetic risk factor of obesity programming and consecutive diabetic and cardiovascular disorders and diseases, in terms of the metabolic syndrome.

Introduction

Overweight is becoming increasingly prevalent even in childhood, for unclear reasons. Overweight and obesity are probably the most important risk factors for the development of type 2 diabetes, the metabolic

syndrome and cardiovascular diseases (Ford *et al.* 2002). Consequently, even the latter at present applies to children and adolescents (Goodman *et al.* 2005). Accordingly, obesity epidemics and subsequent morbidity and mortality are among the major health challenges nearly worldwide.

Environmental alterations during 'critical periods' of development, especially materno-fetal hyperglycaemia, early overfeeding, and rapid weight gain may permanently

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increase the risk of overweight and associated diseases (Plagemann *et al.* 1999*a*; Boullu-Ciocca *et al.* 2005; Plagemann, 2005; Stettler *et al.* 2005; Singhal *et al.* 2007). For this and other phenomena of early imprinting of fundamental life functions and its potential consequences for the development of chronic diseases, the term 'perinatal programming' has been proposed (Dörner, 1975; Plagemann, 2005). Although this concept dates back to the 1970s (Dörner, 1975), it had not been widely recognized until the 'small-baby-syndrome' hypothesis was formulated (Hales & Barker, 1992). Obesity and diabetes, with its metabolic and cardio-vascular consequences following altered pre- and neonatal conditions, have to be regarded as the longest- and best-investigated examples of perinatal programming (Dörner, 1975; Plagemann *et al.* 1999*a*; Plagemann, 2004, 2005; Boullu-Ciocca *et al.* 2005; Gillman, 2005; Stettler *et al.* 2005; Singhal *et al.* 2007).

Rats raised in 'small litters' (SL) are an established animal model in this field. Through artificial reduction of the natural litter size to only three pups per nest SL neonates are subjected to early overfeeding, indicated e.g. by early hyperglycaemia, rapid fat accumulation, and obesity. By unknown mechanisms obesity disposition is preserved permanently, accompanied by hyper-phagia, hyperinsulinaemia, hyperleptinaemia, diabetic and cardiovascular disturbances throughout later life (Plagemann *et al.* 1999*a*; Plagemann, 2005; Boullu-Ciocca *et al.* 2005).

Neuropeptidergic circuits in the mediobasal hypo-thalamus play a key role in the regulation of feeding and body weight (Schwartz *et al.* 2000). Physio-logically, circulating leptin and insulin stimulate in particular the expression of the anorexigenic neuro-hormone proopiomelanocortin (POMC), while inhibiting the orexigenic neuropeptide Y (NPY) (Schwartz *et al.* 2000; Cone, 2005; Horvath, 2005). Early overfeeding leads to permanent dysregulation of these hypothalamic circuits, including functional resistance to insulin and leptin, which may underlie permanently increased food intake and overweight (Plagemann *et al.* 1999*b*; Davidowa & Plagemann, 2000, 2007; Davidowa *et al.* 2003; Plagemann, 2005). Remarkably, similar findings have recently been obtained in offspring of rat dams with diet-induced obesity during pregnancy (Kirk *et al.* 2009). Causal molecular mechanisms are unknown.

Hormones play a pivotal role in the functional and structural organization of feeding circuits (Plagemann, 2004, 2005), as recently shown for the melanocortinergic system (Gao *et al.* 2007). The question remains, however, whether and how such processes of programming might be translated to the genomic level. Epigenetic molecular events leading to permanent alterations in gene expression, such as changes in DNA methylation status (Jirtle & Skinner, 2007), could form a general basis of perinatal programming. For glucocorticoids, it has been shown that environmental influences during neonatal life can induce alterations in central nervous promoter methylation, with lasting consequences for gene expression and consecutive stress response (Weaver *et al.* 2004; McGowan *et al.* 2009). Similarly, neonatal overfeeding might lead to altered methylation of promoter regions of neuro-peptides at central sites critical for the regulation of body weight and metabolism. This could result e.g. in permanent uncoupling of neuropeptide gene expression from circulating leptin and insulin levels, explaining lasting adipogenic and diabetogenic consequences of neonatal overfeeding. We therefore hypothesized that early overnourishment may lead to acquired epigenetic alterations, in terms of altered promoter methylation, of neurohormonal systems involved in feeding, metabolism and body weight regulation (Plagemann, 2005).

To evaluate our hypothesis we investigated whether rats overfed in SL develop alterations in DNA methylation patterns of promoters of genes encoding orexigenic and anorexigenic hypothalamic neuropeptides, thereby potentially contributing to the development of obesity, diabetes and cardiovascular alterations.

Methods

Animal model

The investigations were performed in offspring, bred in our laboratory, of Wistar rats of an outbred colony (CRL:WI; Charles River, Sulzfeld, Germany). All animal procedures were approved by the local animal welfare committee (G 0093/02; LAGETSI Berlin, Germany) and were in accordance with the European Communities Council Directive (86/609/EEC). Virgin female rats were time-mated with normal males at the age of 3 months. On the first day of life, newborns were distributed among the mothers ($n = 13$) randomly, for minimizing confounding by differences in maternal care and lactational performance. To induce neonatal over-nutrition, the primary litter size was then reduced on the third day of life to only three pups per litter (small litters/nests; SL; $n = 8$ litters). In normal litters, the size was adjusted to 12 neonates per mother (normal litters; NL; $n = 5$ litters) (Fiorotto *et al.* 1991; Plagemann *et al.* 1999*a*,*b*; Davidowa & Plagemann, 2003; Boullu-Ciocca *et al.* 2005). The time point of the third day of life was chosen because it has been shown that lactation is fully established then (Fiorotto *et al.* 1991).

All animals were kept under standard conditions with 12 h:12 h inverse light–dark rhythm. They had free access to tap water and standard pellet diet (commercial control diet for rats, energy content: 3.5 kcal g^{-1}, Code 1000; Altromin, Lage, Germany).

Body weight, body fat, blood glucose, insulin and leptin

Body weight was measured on postnatal days (P) 1, 3, 7, 14 and 21 with an accuracy of 0.1 g (scales, Sartorius MC 1, Laboratory LC 6200). Body length was taken on P21 with an accuracy of 0.5 cm. For determination of body fat, carcass mass was measured after stomach and intestine had been removed. Body fat content was evaluated by drying the carcass to constant weight, followed by whole-body chloroform extraction in a Soxhlet apparatus (Schmidt *et al.* 2000).

Trunk blood was collected after rapid decapitation on P21 for determining blood glucose, insulin and leptin. Blood glucose was assayed by photometry using the glucose oxidase–peroxidase method (Dr. Lange GmbH, Berlin, Germany). Within one assay for determination of immunoreactive plasma insulin a modified commercial radioimmunoassay was performed (Adaltis, Freiburg, Germany). Rat insulin (Novo Nordisk Biolabs, Copenhagen, Denmark) with a biological potency of $21.3\,\mathrm{IU\,mg^{-1}}$ was used as standard preparation. The intra-assay coefficient of variation was 4.5–7.4% in a concentration range of 9.1–$94.2\,\mathrm{mIU\,l^{-1}}$. Leptin concentration was measured using a commercial radio-immunoassay (rat leptin RIA kit, Linco). Recombinant rat leptin (Linco) served as the standard preparation. The intra-assay variation was 2.0–4.6% in a concentration range of 1.6–$11.6\,\mu\mathrm{g\,l^{-1}}$. A total of nine rats from five normal litters and nine rats from eight small litters were randomly assigned to this part of the study.

Tissue preparation

Rats were killed on P21 by rapid decapitation. Brains were quickly removed, frozen on dry ice immediately, and then stored at $-70°C$ until subsequent preparation. Preparation of whole hypothalami was performed on a pre-chilled glass slide within a cryostat at $-20°C$. The brain was placed with its ventral surface facing up under a stereomicroscope. Transverse cuts through the caudal optic chiasm and immediately posterior to the mamilliary bodies were performed using a pre-chilled razor blade. By a horizontal cut below the anterior commissure and vertical cuts passing through the perihypothalamic sulci the hypothalami were removed *en bloc* and were sub-sequently analysed in total (Plagemann *et al.* 1998, 1999*b*; McGowan *et al.* 2009).

DNA extraction and sodium bisulfite modification

Dissected tissues were homogenized in Trizol Reagent (Gibco BRL, Invitrogen, Karlsruhe, Germany). Genomic DNA was isolated according to the manufacturer's instructions.

Sodium bisulfite modification, amplification of modified DNA, ligation and cloning were performed as described by Olek *et al.* and McGowan *et al.* with slight modifications (Olek *et al.* 1996; Harder *et al.* 2004; McGowan *et al.* 2009). The method of sodium bisulfite modification was chosen for detecting 5′-methylcytosines because it has been demonstrated to be the most sensitive method of detecting CpG methylation (Clark *et al.* 2006). While treating fragmented DNA with sodium metabisulfite, unmethylated cytosines are converted to uracil residues by deamination. During PCR both uracil and thymine are amplified as thymine.

Bisulfite modification

The bisulfite reaction was carried out in $3\,\mu\mathrm{g}$ of linear fragmented DNA after digestion with the restriction enzyme *Rsa* I. Following ethanol-induced DNA precipitation, DNA was denatured by $1\,\mathrm{M}$ NaOH and embedded in agarose beads (2% low melting point agarose) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Sodium metabisulfite (Sigma-Aldrich Chemie GmbH), to a final concentration of $2.5\,\mathrm{M}$, and hydro-quinone (Sigma-Aldrich Chemie GmbH), to a final concentration of $125\,\mathrm{mM}$, pH 5.0, were added to agarose beads and incubated at $50°C$ for 5 h. Reaction was stopped by $1\times$ Tris/HCl/ethylenediamine tetra-acetic acid (EDTA); Tris (Merck, Darmstadt, Germany), HCl (Merck), EDTA (Sigma-Aldrich Chemie GmbH). Purification of agarose beads was performed as previously described (Harder *et al.* 2004).

Selection of NPY promoter and POMC promoter fragments

Using all currently available literature and gene data bank information, we selected for investigations a 246 base-pair fragment of the NPY promoter (Genbank accession no.: M15792). According to the definition by Gardiner-Garden & Frommer (1987), the rat NPY promoter is located in a CpG island (Larhammar *et al.* 1987). The NPY fragment investigated here $(-246 - -1)$ included the minimal promoter of the NPY gene (Larhammar *et al.* 1987; Minth & Dixon, 1990; Minth-Worby, 1994). The fragment covered all currently known consensus sequences of major transcription factor binding sites. These include the binding sequences of nerve growth factor (NGF), activator protein 1 (AP-1), activator protein 2α (AP-2α), nerve growth factor-induced gene A (NGFI-A) and specific protein 1 (Sp1) (Minth & Dixon, 1990; Higuchi *et al.* 1992; Minth-Worby, 1994; Lerchen *et al.* 1995; Li *et al.* 2000; Shimizu-Albergine *et al.* 2001). Analogously, a

404 base-pair fragment of the POMC promoter was investigated (Genbank accession no.: M74296). The rat POMC gene promoter is also located in a CpG island (Newell-Price *et al.* 2001). The POMC promoter fragment investigated here spans the tissue-specific 5′CpG-island which was described for the human POMC promoter to be differently methylated in various tissue types (Newell-Price *et al.* 2001) and includes all DNA binding sequences of regulatory elements that were characterized to be relevant for transcriptional expression of the POMC gene. This included the consensus sequences of nuclear factor κB (NF-κB), Sp1, signal transducer and activator of transcription 3 (STAT 3), negative glucocorticoid receptor element (nGRE) and forkhead box O1 (FoxO1) (Therrien & Drouin, 1991; Drouin *et al.* 1993; Jin *et al.* 1994; Liu & Mortrud, 1995; Mynard *et al.* 2002; Kitamura *et al.* 2006).

Amplification of promoter regions, ligation, cloning and sequencing

Bisulfite-modified DNA embedded in agarose beads was amplified either with the forward primer NPY (-290 bp/-264 bp): 5′-TTT TTG TTT TAT TTT TTT TTT TGG TAG-3′ and the reverse primer NPY ($+83$ bp/$+105$ bp): 5′-TCC CAA TTA ATC CTA ACA CTC AC-3′ (annealing 54°C, 45 cycles) or with the forward primer POMC (-540 bp/-515 bp): 5′-GTT TTG GGT TGT TAT GAT TTT TGA T-3′ and the reverse primer POMC (-1 bp/-23 bp): 5′-AAT CCC TAT CAC TCT TCT CTC TT-3′ (annealing 58°C, 40 cycles). Genomic fragments of NPY promoter of 395 bp and of POMC promoter of 540 bp were amplified and purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Amplified and purified DNA was ligated into the vector pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany) and transformed into chemically competent cells *E. coli TOP10F′* (Invitrogen), according to the manufacturer's instructions of TOPO TA Cloning Kit (Invitrogen). Plasmids were plated on media containing ampicillin (Sigma-Aldrich Chemie GmbH), X-gal (Bio Vectra, Charlottetown, Canada) and IPTG (Sigma-Aldrich Chemie GmbH) and were incubated overnight. Transformants were assayed for the presence of recombinant inserts by the blue/white colony phenotype and plasmids were extracted with QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Inserts were controlled with restriction enzyme *Bst*x I (Promega, Mannheim, Germany). The inserts in plasmids were amplified with the forward primer pUC/M13: 5′-GTT TTC CCA GTC ACG AC-3′ and the reverse primer pUC/M13: 5′-CAG GAA ACA GCT ATG AC-3′ (annealing 50°C, 35 cycles). Purification was performed with the QIAquick PCR Purification Kit (Qiagen) according to the

manufacturer's instructions. The NPY fragment included nucleotides -246 to -1, the POMC fragment nucleotides -404 to -1 of their respective primary promoter.

Eleven to eighteen clones per animal were sequenced for each promoter (Genetic Analyzer 3100; Applied Biosystems, Darmstadt, Germany) using the pUC/M13 reverse primer and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Applied Biosystems) according to the manufacturer's instructions. A total of eight rats from five normal litters and eight rats from eight small litters were randomly assigned to this part of the study.

RNA extraction, cDNA synthesis and RT-PCR

In hypothalami prepared as described above, the arcuate hypothalamic nucleus (ARC) was dissected (Plagemann *et al.* 1999b) and mRNA was isolated by TRIZOL Reagent (GibcoBRL, Invitrogen) according to the manufacturer's instructions. RNA (3 μg) was reverse transcribed into complementary DNA (cDNA) using the reagents provided in the SuperScript First-Strand Synthesis System (Invitrogen) for RT-PCR according to the manufacturer's instructions. Primers were designed spanning an intron, POMC forward (5′-GAG ATT CTG CTA CAG TCG CTC-3′) and POMC reverse (5′-TTG ATG ATG GCG TTC TTG AA′) (Millington *et al.* 1999). The cDNA was amplified by an annealing temperature of 59°C and 30 cycles. To get a measure of POMC expression relative to the individual leptin or insulin level of each animal, POMC expression was divided by leptin and insulin level, respectively, and presented as relative POMC expression (log a.u. (arbitrary units)). A total of eight rats from five normal litters and eight rats from eight small litters were randomly assigned to this part of the study (same animals as for the methylation part).

Immunocytochemistry

After rapid decapitation brains were removed and fixed in Bouin's solution for 48 h. After embedding, 5 μm-thick serial coronal sections were cut through the hypothalamus at planes 24–32, corresponding to bregma levels -1.40 to -3.60 mm (Paxinos & Watson, 1986). Alternate slides were stained with cresyl violet (Nissl+), or immunostained for POMC, using the avidin–biotin–peroxidase complex (ABC) method (Vectastain Kit; Vector Laboratories, Burlingame, CA, USA). For immunocytochemistry, slides were pretreated with Triton X-100 (Ferak, Berlin, Germany) followed by incubation in methanol containing 0.3% hydrogen peroxide and rinsed with 2% normal horse serum (Vector). Incubation with a rabbit antibody to rat POMC (concentration 1:5000; Phoenix, Belmont, CA,

USA) was performed for 48 h in a humid chamber at 4°C. After washing, slides were treated with biotinylated anti-mouse IgG (1:500; Vector) and then incubated with ABC for 2 h. Sections were exposed to a 0.05% solution of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) for 20 min. Specificity of the labelling procedure was verified by the absence of immunocytochemical reaction in sections in which the primary antibody was omitted or was substituted by normal serum. In each animal, the complete cranio-caudal extention of the ARC was covered by stained sections.

Neuromorphometry

Morphometric analysis within the ARC was performed using an image analysing system (KS 400 V.3.0; Zeiss, Jena, Germany) connected by a video camera (DXC-390P; Sony, Japan) to a light microscope (Axioscope; Zeiss). Using successive serial sections, percentage immuno-positivity of POMC+ neurons was analysed within the ARC at a final magnification of ×1700 in both hemispheres of the brain by one investigator, without knowledge of the experimental group. Only neurons with a distinct nucleolus and soma appearance were included in the measurement (Plagemann *et al.* 1998, 1999*a,b*; Fahrenkrog *et al.* 2004; Franke *et al.* 2005). A total of five rats from five normal litters and six rats from six small litters were used for this part of the study.

Data analysis

For data analysis of sequences the software SeqManII (DNASTAR, Madison, WI, USA) was used. Semi-quantitative detection of mRNA concentration was performed using the fluorescence-imager Typhoon 8600 (Amersham Biosciences Europe GmbH, Freiburg, Germany) and the software Image Quant (Amersham Biosciences Europe GmbH).

Methylation status was analysed for every single cyto-sine position of the NPY promoter and the POMC promoter, respectively, using the approach suggested by Siegmund & Laird (2002) and Meaney and colleagues (Weaver *et al.* 2004; McGowan *et al.* 2009): The number of methylated cytosines for each cytosine residue was summed up and divided by the total number of clones. This procedure was repeated for each animal. The resulting proportion of methylated cytosines per position is given as a percentage of all cytosine residues in all animals (Siegmund & Laird, 2002; Weaver *et al.* 2004; McGowan *et al.* 2009). For statistical analyses, in all variables distribution was tested for normality. In the case of significant non-normality (insulin levels), the values were log-transformed for statistical tests. Group differences were compared using Student's *t* test.

To further evaluate the robustness of the key results, two additional statistical approaches were applied to the methylation data. First, we corrected for multiple testing using the Bonferroni correction. Second, to account for the hierarchical structure of the experiment, i.e. the fact that pups are nested within a litter and therefore do not represent independent measurements (although randomly assigned), additionally mixed-model (multilevel) analyses were performed using litter as the higher-ranking hierarchical level. For correlation Spearman's rank correlation test was used. Data analysis was performed by SPSS (Statistical Package for the Social Sciences for Windows 11.0; SPSS Inc. Munich, Germany).

Results

Effect of neonatal overnutrition on body weight, body fat content and metabolic parameters

Rats exposed to overfeeding by rearing in SL rapidly developed overweight from P7 onwards ($P < 0.001$; Fig. 1*A*). At P21, body weight was highly significantly increased in SL rats, as compared to controls ($P < 0.001$; Fig. 1*A*). Analysis of body composition showed that this was due to a nearly doubled percentage of body fat in SL rats ($P < 0.001$; Fig. 1*B*). Obesity was accompanied by hyperleptinaemia ($P < 0.001$), hyper-glycaemia (7.5 ± 0.27 mmol l^{-1} *vs.* 8.3 ± 0.17 mmol l^{-1}; $P < 0.05$) and hyperinsulinaemia ($P < 0.001$; Fig. 1*C* and *D*). Furthermore, SL rats had a significantly increased insulin/glucose ratio as an indicator of insulin resistance (3.7 ± 0.26 IU mol^{-1} *vs.* 8.3 ± 0.72 IU mol^{-1}; $P < 0.001$). Thus, characteristic features of the metabolic syndrome occurred already at weaning in infantile SL animals.

DNA methylation pattern of the hypothalamic NPY gene promoter in neonatally overfed rats

Against the background of this acquired phenotype, using bisulfite sequencing as the 'gold-standard' technique we mapped the methylation status of cytosines within CpG dinucleotides of the NPY promoter in hypothalamic tissue. The NPY promoter spans over 246 base pairs including 17 CpG dinucleotides (Larhammar *et al.* 1987). Among the five transcription factor binding sites which were covered by the sequence (NGF-RE, Sp1, AP-1, AP-2, NGFI-A), only those for NGF-RE, AP-2 and NGFI-A included CpG dinucleotides and were therefore potentially accessible to DNA methylation (Fig. 2*A*). Analysis of single CpG residues showed that the NPY promoter was methylated at low levels in both SL and NL rats: in none of the positions were more than 5% of CpGs found to be methylated (Fig. 2*B*). The analysis of the three

CpG-containing transcription factor binding sites showed no significant group differences (Fig. 2*C*).

DNA methylation pattern of the hypothalamic POMC gene promoter in neonatally overfed rats

Using the same technique, we then mapped the methylation status of CpG dinucleotides within the hypothalamic POMC promoter. The POMC promoter spans over 404 base pairs including 21 CpG dinucleotides (Newell-Price *et al.* 2001). The sequence covers four activating transcription factor binding sites (FoxO1, STAT3, Sp1, NF-κB) and one inhibiting transcription factor binding site (nGRE). However, only those for NF-κB and nGRE contain CpG dinucleotides and are therefore accessible for methylation. Whereas the Sp1 binding site itself does not contain CpGs, it is known that methylation of the region upstream of this sequence decisively influences Sp1-induced gene transcription (Zhu *et al.* 2003). Therefore, we additionally investigated this region (Fig. 3*A*).

Compared to the NPY promoter, the POMC promoter showed considerably higher levels of DNA methylation. Analysis of single dinucleotides showed that upstream parts of the promoter generally were more strongly methylated in SL rats, as compared to control animals, while, to the contrary, more downstream CpGs tended to be less methylated in SL (Fig. 3*B*). To quantify

these results and to evaluate a potential impact of these group differences on POMC gene expression, we calculated the levels of DNA methylation within the three CpG-containing transcription factor binding sites and regions. Binding sequences of the activating transcription factor NF-κB ($P < 0.05$) and the region upstream of the Sp1 binding site showed significantly increased methylation in SL rats, as compared to controls ($P < 0.05$; Fig. 3*C*). In contrast, the inhibiting nGRE binding sequence showed a non-significantly decreased methylation in SL rats (Fig. 3*C*). The significance of increased methylation of the NF-κB/SP1 region was confirmed after Bonferroni correction ($P < 0.05$). The level of significance was even slightly higher for both regions when litter origin was accounted for by multilevel analysis ($P < 0.01$).

Relations between metabolic parameters and POMC promoter methylation

To evaluate the hypothesis that metabolic alterations due to neonatal overfeeding could be responsible for increased promoter methylation in SL rats, we performed correlation analyses between metabolic parameters and POMC promoter methylation. Remarkably, blood glucose levels showed a significant positive correlation to the degree of POMC promoter methylation ($r = 0.63$; $P = 0.01$; Fig. 4*A*). The absence of significant correlations
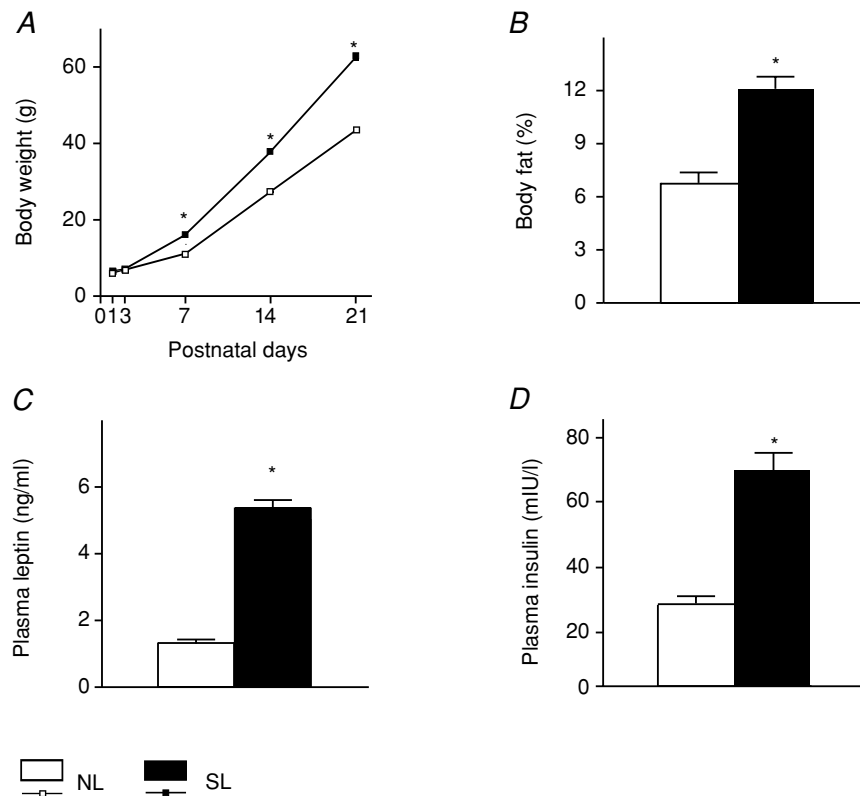

Figure 1
Body weight development (*A*), and body fat (*B*), plasma leptin (*C*) and insulin (*D*) in rats raised in small litters (SL) or normal litters (NL; controls) on P21 (*n* = 9 animals/group). *$P < 0.001$ (unpaired *t* test). Data are means ± S.E.M.

to leptin ($r = 0.12$; $P = 0.65$) and insulin ($r = 0.32$; $P = 0.24$) indicated that this was not an unspecific finding caused by group differences in the respective parameters.

Consequences of increased POMC promoter methylation for leptin- and insulin-dependent hypothalamic POMC gene expression

We then investigated whether increased methylation of activating transcription factor binding sites might have had consequences for hypothalamic POMC gene expression. Despite 2- to 3-fold increased leptin and insulin levels in SL rats (Fig. 1), which normally would lead to a strong increase in POMC expression

(Brown *et al.* 2006; Sahu, 2008), hypothalamic POMC expression in SL showed no increase, as compared to NL rats (NL: 0.64 ± 0.03 a.u. *vs.* SL: 0.55 ± 0.05 a.u.; $P = 0.13$). Additionally, we replicated this finding of a lack of upregulation of POMC on the protein level, showing by immunocytochemistry that POMC immunopositivity did not differ quantitatively between hyperleptinaemic/hyperinsulinaemic SL rats and normoleptinaemic/normoinsulinaemic NL rats (NL: 362 ± 37 POMC+ mm^{-2} *vs.* SL: 395 ± 6.8 POMC+ mm^{-2}; $P = 0.42$).

Because of the well-known dependency of POMC expression on circulating leptin and insulin (Schwartz *et al.* 2000; Cone, 2005), we additionally calculated the quotient of POMC expression per unit of
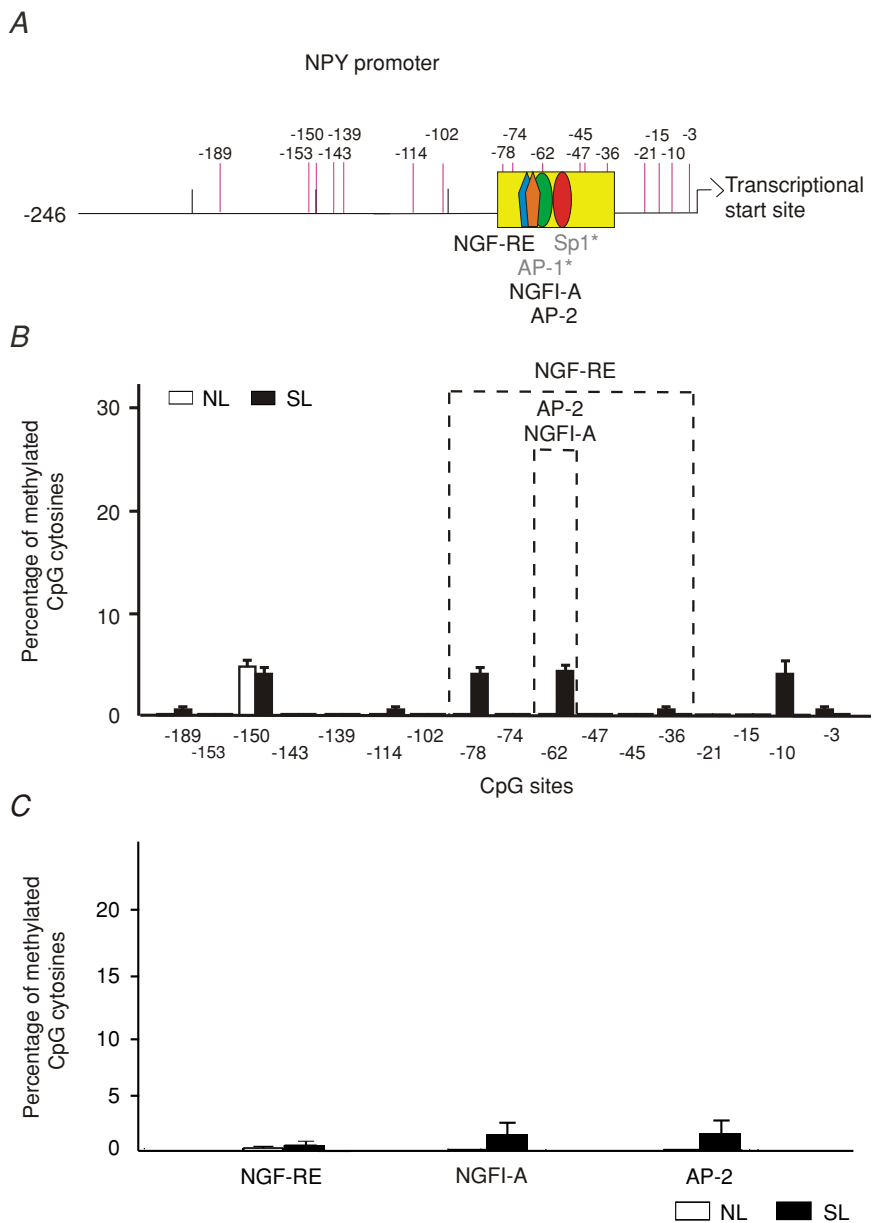

Figure 2
A, sequence map of the neuropeptide Y (NPY) gene promoter region including functionally regulatory elements (boxed) and CpG dinucleotides (longer lines). *The binding sequences of Sp1 and AP-1 do not contain CpG dinucleotides and are therefore not accessible to DNA methylation. *B*, methylation analyses of the hypothalamic NPY promoter from small litter (SL) and normal litter (NL; controls) rats. *C*, percentage of CpG residues that were methylated within the CpG-containing binding sequences for nerve growth factor regulatory element (NGF-RE; −78 to −36), nerve growth factor-induced gene A (NGFI-A; −62) and activator protein 2 (AP-2; −62) within the NPY promoter region. Group differences were not statistically significant ($P > 0.05$ *t* test with Bonferroni correction; $P > 0.05$ after adjustment for litter origin by multilevel analysis). Data are means ± S.E.M. (11–18 clones sequenced per animal; $n = 8$ animals/group).

leptin and insulin, respectively. In SL rats, POMC expression was clearly decreased per corresponding leptin $((131 \pm 29) \times 10^3$ a.u. *vs.* $(35 \pm 3) \times 10^3$ a.u.; $P = 0.01)$ and insulin $((47 \pm 5.8) \times 10^2$ a.u. *vs.* $(27 \pm 3.2) \times 10^2$ a.u.; $P = 0.01)$.

Given that POMC expression is regulated decisively by leptin and insulin, it is not reasonable to correlate the extent of promoter methylation to POMC expression without considering the actual levels of leptin and insulin. We therefore finally used the above-described integrative parameters 'POMC expression per unit leptin' and 'POMC expression per unit insulin' to evaluate whether the extent by which hypothalamic POMC expression is regulated by leptin and/or insulin might have depended on the level of neonatally acquired POMC promoter methylation. To do so, we correlated the individual percentage of methylation

upstream of the activating Sp1 binding site to POMC expression per unit of leptin and insulin, respectively. This analysis revealed significant inverse correlations between the extent of DNA methylation at this promoter site and POMC expression per unit of leptin (Fig. 4*B*) and insulin (Fig. 4*C*). That is, the higher the degree of methylation was, the smaller the leptin- and insulin-induced upregulation of hypothalamic POMC expression.

Discussion

This is the first study to demonstrate an epigenetic 'programming' effect of an altered nutritional milieu on a central nervous system regulating feeding and body weight control, affecting overweight disposition and thereby the
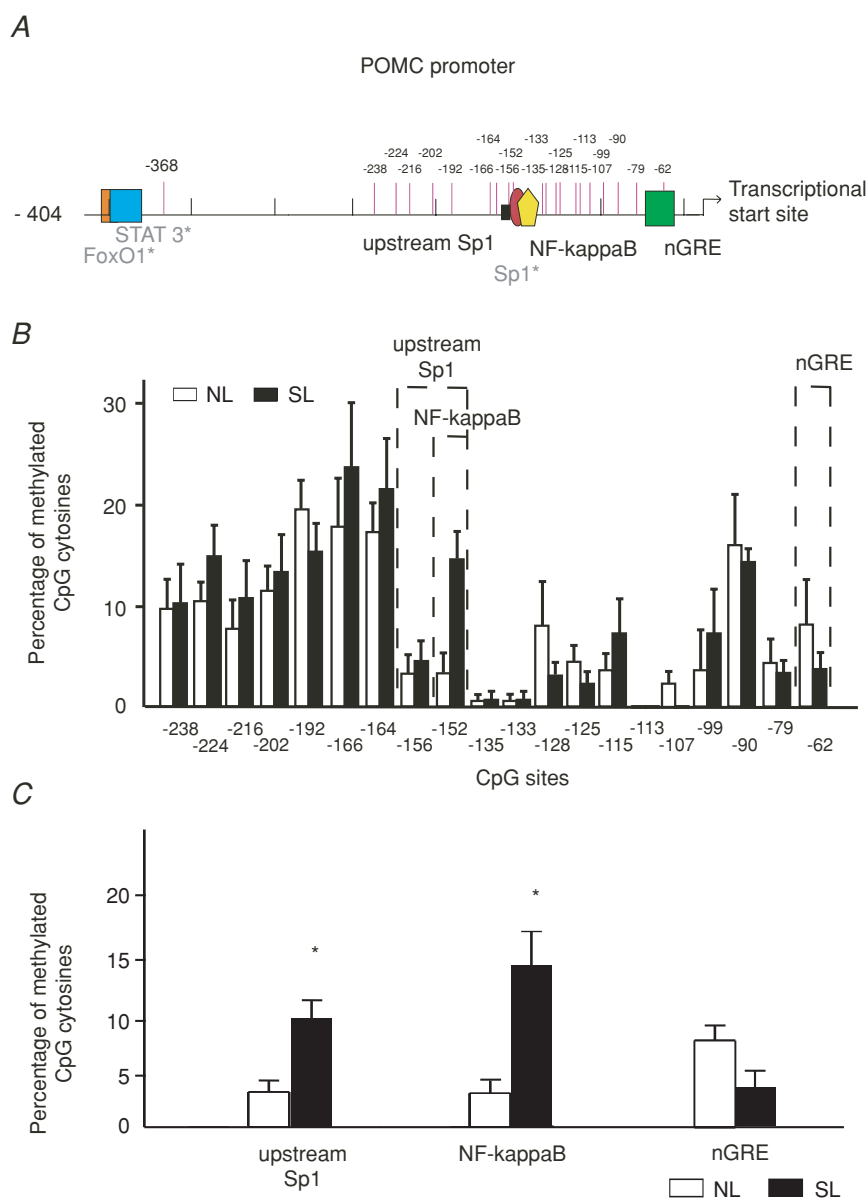

Figure 3
A, sequence map of the proopiomelanocortin (POMC) gene promoter region including functionally regulatory elements (boxed) and CpG dinucleotides (longer lines). *The binding sequences of FoxO1, STAT3 and Sp1 do not contain CpG dinucleotides and are therefore not accessible to DNA methylation. *B*, methylation analyses of the hypothalamic POMC promoter from small litter (SL) and normal litter (NL; controls) rats. CpG at position −368 was methylated at 84% in both groups (NL: $84 \pm 4.3\%$ *vs.* SL: $84 \pm 2.6\%$) and is therefore not displayed to keep the scale of the *y*-axis. *C*, percentage of CpG residues that were methylated within the CpG-containing binding sequences for Sp1 (upstream Sp1; −156 to −15), nuclear factor-κB (NF-κB; −152) and negative glucocorticoid receptor element (nGRE; −62) within the POMC promoter region. *$P < 0.05$ (*t* test with Bonferroni correction; $P < 0.01$ after adjustment for litter origin by multilevel analysis). Data are means $\pm$ s.e.m. (11–18 clones sequenced/animal; $n = 8$ animals per group).

risk of subsequent diabetic and cardiovascular disorders and diseases in terms of the metabolic syndrome.

The methylation pattern of the promoter of NPY is characterized here for the first time in any species and tissue, in general. NPY, known to be the most potent orexigenic neurohormone, particularly acts within the arcuate-paraventricular hypothalamic axis. This hypothalamic system consists of neurons expressing NPY in the arcuate nucleus which project to the paraventricular nucleus. The expression and release of NPY is regulated by circulating insulin and leptin (Schwartz *et al.* 2000). In a previous study, we observed that the hypothalamic expression of NPY is not decreased in SL rats at weaning, despite largely increased levels of insulin and leptin (Plagemann *et al.* 1999*b*), whereas another group even observed a paradoxical increase of hypothalamic NPY expression in SL rats (Lopez *et al.* 2005). However, in contrast to our expectations, we did not observe a significant group difference in DNA methylation within the hypothalamic NPY promoter here. Rather, the analysis revealed global hypomethylation in all animals, i.e. independent of the mode of early nutrition. Our data might therefore suggest a genuine resistance of the NPY promoter to environmentally induced modifications. This seems plausible, considering NPY as the most powerful orexigenic neuropeptide, which is vitally important for the individuum and therefore highly conserved in different species (Larhammar *et al.* 1987). Thus, one may speculate that an epigenomic 'fine-tuning' of food intake and body weight regulation is rather realized via anorexigenic than orexigenic mechanisms. This would make sense, even from an evolutionary perspective, to ensure individual survival by adequate fuel supply as a primary and overall purpose.

The anorexigenic melanocortinergic system has been suggested to be the best-characterized hypothalamic circuit involved in the regulation of energy balance (Cone, 2005). In the hypothalamus, POMC, the precursor molecule of the post-translational cleavage peptide α−melanocyte-stimulating hormone which acts via the melanocortin-4 receptor in the paraventricular nucleus, is synthesized exclusively in the ARC (Cone, 2005). An increase in POMC expression in the ARC reduces food intake and decreases body weight, while the expression, synthesis and release of POMC are physiologically increased in the presence of elevated leptin and insulin, which thereby act as circulating satiety signals (Schwartz *et al.* 2000; Cone, 2005). In this context, it is important to note that POMC expression has been shown to be strongly influenced by promoter methylation (Newell-Price *et al.* 2001). Our study is the first one to investigate POMC promoter methylation in the hypothalamus. A comparison with the literature reveals that methylation levels within the hypothalamus are lower than in non-POMC-expressing tissues, such as lung, but higher and more variable than in other POMC-expressing organs, such as pituitary (Newell-Price *et al.* 2001).

We show here that both of the two CpG-containing binding sites which activate hypothalamic POMC expression become hypermethylated through overfeeding. Sp1 is a major activator of POMC transcription (Therrien & Drouin, 1991). It has recently been shown that activation of the Sp1 binding site in the POMC promoter is essential for the mediation of leptin effects on POMC expression (Yang *et al.* 2009). The expression of Sp1 is physiologically stimulated by insulin (Pan *et al.* 2001). It is important to note that instead of methylation of the
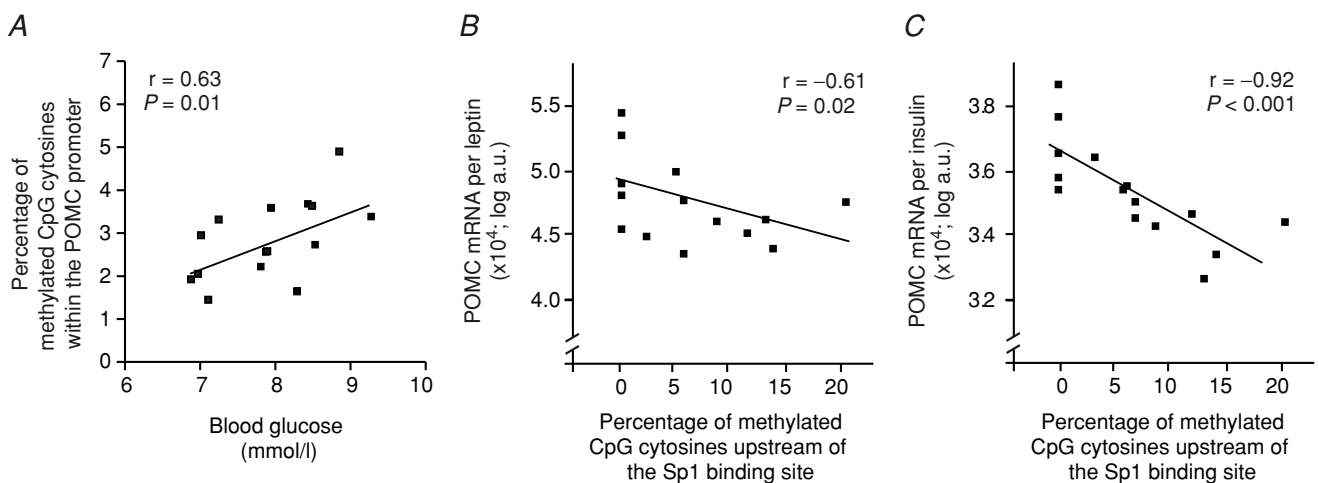

Figure 4
Hypothalamic POMC promoter methylation, presented as a function of neonatal blood glucose levels (*A*), and hypothalamic POMC expression calculated per corresponding level of leptin (*B*), and insulin (*C*), presented as a function of the percentage of CpG residues that are methylated upstream of the SP1 binding sequence of the POMC promoter. Correlation coefficients and significances derived from Spearman rank correlation test.

Sp1 binding site itself, methylation upstream of this sequence has been shown to inhibit Sp1 binding (Zhu *et al.* 2003). Hypermethylation upstream of the Sp1 binding sequence, as observed here, may therefore impair POMC expression in hyperinsulinaemic SL rats in terms of acquired 'silencing' at this site. On the other hand, Sp1 can also bind to the NF-κB site. However, this partly overlapping binding sequence also became significantly hypermethylated in SL rats. Moreover, simultaneously the only inhibitory regulator of POMC expression, the nGRE binding site (Drouin *et al.* 1993), was not normally or hyper-methylated but, on the contrary, was found to be even less methylated, thereby potentially enhancing inhibition of POMC transcription. This complex of specific alterations with varying directions strongly suggests functionality of acquired methylation pattern.

We therefore tested whether increased methylation of activating transcription factor binding sites had consequences for gene expression. It is important to consider that the hypothalamic POMC expression is highly dynamically influenced by the circulating levels of leptin and insulin. In the presence of high leptin and insulin, POMC expression increases (Brown *et al.* 2006; Sahu, 2008). In neonatally overnourished SL rats, however, marked hyperinsulinaemia and hyperleptinaemia were not accompanied by an increased expression of POMC, as one would expect from the physiology of regulation (Schwartz *et al.* 2000; Cone, 2005; Brown *et al.* 2006; Sahu, 2008). Rather, neither POMC mRNA expression nor POMC immunopositivity in the ARC, i.e. the hypothalamic expression locus of POMC (Cone, 2005), differed between overfed and control rats. This lack of upregulation of the major anorexigenic neurohormone despite the presence of hyperleptinaemia and hyperinsulinaemia clearly indicates a neonatally acquired leptin and insulin resistance, as it was functionally demonstrated during previous studies (Davidowa & Plagemann, 2000, 2007).

In our study, methylation patterns were obtained using bisulfite sequencing. Although this method is regarded to be the 'gold standard' technique for DNA methylation analysis (Clark *et al.* 2006), one has to consider the potential limitations of this approach. One of them could be PCR bias. This term is used in the literature to describe a potential pitfall during the conduct of methylation analysis whereby certain sequences may be amplified preferentially during PCR, leading to an inaccurate estimate of methylation (Warnecke *et al.* 1997). To control for PCR bias, particular attention must be given to the design of PCR primers for the methylation study. Recently, Wojdacz *et al.* (2008) have suggested a list of five criteria of PCR primer design which have to be met to avoid PCR bias, e.g. melting temperature limits and conduct of specific control PCRs. All of these criteria were met in our study in both cases of promoter analysis, thereby strongly limiting the possibility of PCR bias.

To evaluate the potential limitations of our study regarding the number of clones and animals used, we made two calculations of its statistical power. We first calculated the power of our study to detect a significant result at the level of $P < 0.05$. Given eight animals in each group (NL, SL) and standard deviations which are 50% of the mean, our study had a power of 91.5% to detect at least a doubling of the methylation level. Secondly, we aimed to calculate how many clones in each animal would be necessary to achieve a stable estimate of methylation for a single animal. Given the assumption that the between-animal variation in methylation within a group would not be larger than the between-group variation in methylation, i.e. fivefold in the case of the NF-κB site, one could go down in number of clones picked in each animal to seven clones from which significant differences in methylation between animals from the same group at the level of $P < 0.05$ (power: 80%) could be shown. To summarize, these calculations indicate that our study had a great enough statistical power to reveal significant as well as stable results.

Another potential methodological limitation of our study relates to the fact that our hypothalamic tissue samples used for methylation analysis contained various cell types. Therefore, the observed differences in methylation pattern could theoretically also be caused by a shift in the spatial/number distribution of different cell types which could be differentially methylated. However, while DNA methylation pattern is regarded to be organ-specific, it is not established that methylation differences exist between different cell types within one single organ. Accordingly, in none of the most prominent studies on DNA methylation in models of perinatal programming was the specific type of cells from which the DNA probes came considered (Waterland & Jirtle, 2003; Weaver *et al.* 2004; Bogdarina *et al.* 2007; Park *et al.* 2008; Lillycrop *et al.* 2008; McGowan *et al.* 2009).

We found an indication that the specifically altered methylation pattern acquired due to overfeeding is functionally relevant for subsequent mRNA expression. Usually, it could be expected that increased promoter methylation leads to decreased absolute mRNA levels of the respective gene. This scenario is likely in cases where the expression of the respective gene is relatively stable, as in tumour suppressor genes, but does not largely depend on factors which dynamically regulate gene expression. However, it has to be considered that hypothalamic POMC gene expression decisively depends on circulating leptin and insulin. Both signals from the periphery are known to regulate gene expression of POMC in a dose-dependent manner, especially by activating the SP1 binding site of the promoter (Yang *et al.* 2009). Therefore, from a physiological point of view the level of promoter methylation cannot be directly related to gene transcription, but must be put into the actual, cybernetic context of the given leptin and insulin levels of the

individual animal. This has to be considered necessarily, because together with the level of methylation of the transcription factor binding sites the actual concentrations of leptin and insulin produce the 'net effect' of POMC gene transcription. We therefore calculated a measure of POMC expression in relation to leptin and insulin to account for this. The degree of methylation upstream of the Sp1 binding site in the POMC promoter was significantly inversely correlated to POMC expression per unit of leptin and insulin, respectively. Although this correlation does not necessarily indicate causality, the results show that altered promoter methylation is related to a functional 'uncoupling' of POMC expression from actual circulating leptin and insulin, indicated by failure to upregulate POMC. Thus, overnutrition appears to result in an epigenetically acquired alteration of the regulatory 'set point' of POMC, with 'normal' expression occurring only in the presence of hyperinsulinaemia and hyper-leptinaemia, indicating an acquired malprogramming.

The question remains *how* overfeeding may induce these alterations. Three potential mechanisms should be proposed here. Firstly, hyperinsulinism, as occurring due to overnutrition, induces peripheral as well as central nervous insulin resistance (Plagemann *et al.* 1999*b*; Davidowa *et al.* 2003; Plagemann, 2005; Davidowa & Plagemann, 2007), which leads to decreased Sp1

levels and therefore increased accessibility of the Sp1 binding site to methyltransferases, possibly leading to increased methylation. This idea is supported by positive correlations between the insulin/glucose ratio, an indicator of insulin resistance, and the percentage of methylated CpG residues upstream of the Sp1 binding site ($r = 0.62$; $P = 0.01$) as well as the NF-κB binding site ($r = 0.52$; $P = 0.04$). Secondly, hyperglycaemia has recently been shown to increase DNA-methyltransferase activity and subsequent DNA methylation, leading to glucose-dependent hypermethylation (Chiang *et al.* 2008). Accordingly, in our study glucose levels were found to be significantly positively correlated to the percentage of methylated POMC promoter cytosines. This even occurred for the constitutionally hypo-methylated NPY promoter ($r = 0.59$; $P = 0.02$), strongly supporting causality of the relationships. Since central insulin resistance as well as hyperglycaemia, both typically accompanying overfeeding, are crucial features of the metabolic syndrome, a combination of these two alterations seems to be a particularly plausible and potentially even additive mechanism of acquired epigenetic alterations. Thirdly and finally, in overfed SL pups corticosterone has been shown to be increased (Boullu-Ciocca *et al.* 2005). Subsequently, increased binding of activated glucocorticoid receptors at the
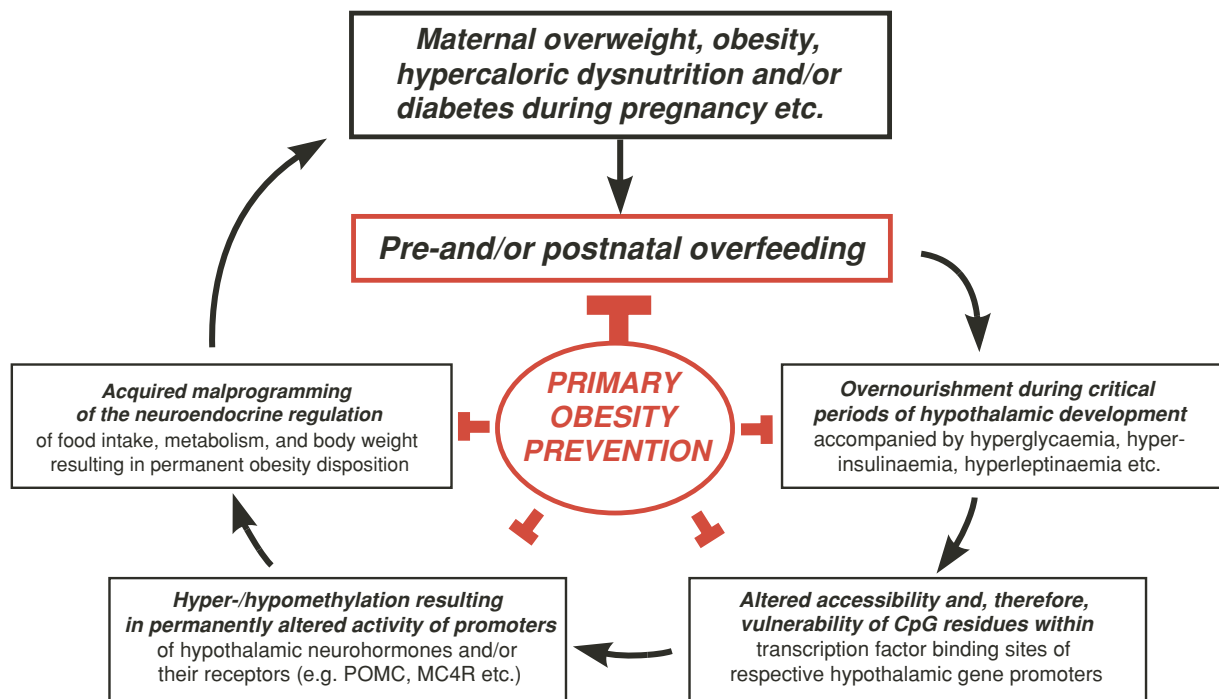

Figure 5

Proposal of a general concept of an intergenerative *'epigenetic vicious circle'* of obesity programming during critical periods of pre- and early postnatal development, caused by permanently altered 'set points' of neurohormone and/or receptor expression in neuro-hormonal circuits regulating food intake, metabolism and body weight. Prevention appears to be possible by normalization of food supply in early life.

nGRE binding site may therefore decrease its accessibility during early development, consequently resulting in hypomethylation. This mechanism seems the more plausible as glucocorticoid-depending alteration of DNA methylation is one of the best investigated mechanisms of epigenetic programming, in general (Weaver *et al.* 2004; McGowan *et al.* 2009). Finally, it should be noted that the mechanisms proposed here may even explain specificity of the acquired alterations observed.

Summarizing, this is the first study revealing nutritionally induced alterations of the methylation pattern within a gene promoter that is critical for body weight regulation and, hence, may be directly relevant for the programming and pathogenesis of obesity and its diabetic and cardiovascular endpoints. Alterations were induced during a critical period of postnatal development, i.e. far beyond the established period of epigenomic reprogramming in early embryonic stages. Specificity of affected transcription factor binding sites, varying direction of altered methylation pattern (i.e. hyper- or hypomethylation) as well as impaired gene expression demonstrate functionality of the acquired alterations.

In conclusion, we would like to postulate here a general new aetiological concept of nutritional *'epigenetic obesity programming'* (Fig. 5). Overfeeding during critical periods of hypothalamic development, especially in the case of maternal gestational overweight/diabetes or neonatal overfeeding, as shown here exemplarily for the SL model, is proposed to lead to an altered accessibility or 'vulnerability' of CpG dinucleotides within promoters of hypothalamic genes involved in the regulation of food intake and body weight. The resulting alterations of the methylation pattern of the respective promoters will ultimately change their activity and consecutive gene transcription. Consequently, the respective regulatory system will become malprogrammed, leading to an increased disposition towards obesity and the metabolic syndrome. If female offspring affected in that way enter reproductive age and become pregnant, by being obese and/or diabetic during pregnancy they expose their offspring in a similar way to a hyper-caloric/hyperglycaemic perinatal environment that they were exposed to themselves, thereby closing an epigenetic 'vicious intergenerative circle'. This mechanism might contribute substantially to the epidemics of obesity and 'diabesity' but, most of all, seems to be preventable by normalization of food supply in early life.

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Author contributions

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