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High fat diet induces hypermethylation of the hypothalamic *Pomc* promoter and obesity in post-weaning rats

Asaf Marco [a,b], Tatiana Kisliouk [c], Aron Weller [b,d], Noam Meiri [c,*]

[a] *Faculty of Life Sciences, Bar Ilan University, Ramat-Gan 52900, Israel*
[b] *Gonda Brain Res Center, Bar Ilan University, Ramat-Gan 52900, Israel*
[c] *Institute of Animal Science, ARO, The Volcani Center, PO Box 6, Bet Dagan 50250, Israel*
[d] *Department of Psychology, Bar Ilan University, Ramat-Gan 52900, Israel*

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Summary Impaired response of the brain to the leptin signal leads to a persisting dysregulation of food intake and energy balance. High plasma leptin or insulin should activate proopiomelanocortin (POMC), the precursor of the anorexigenic neuropeptide α-melanocyte-stimulating hormone (α-MSH) in the hypothalamic arcuate nucleus (ARC). Nevertheless, in obesity, this signal transduction pathway might be impaired. In this study we investigated whether chronic high fat (HF) diet consumption from post-weaning to adulthood increases CpG methylation of the *Pomc* promoter. The hypothesis that this would disrupt the essential binding of the transcription factor Sp1 to the *Pomc* promoter was tested. Male rats were raised from postnatal day 21 till 90 on either HF or standard diet. As a result HF fed rats were significantly heavier, with high leptin and insulin levels in their plasma but almost no changes in ARC mRNA expression levels of *Pomc*. The *Pomc* promoter area in the HF-treated rats was found to be hypermethylated. Furthermore, there was a direct correlation in individual rats between CpG methylation at specific sites that affect Sp1 binding and plasma leptin levels and/or body weight. Although, as expected the HF diet resulted in up-regulation of Sp1, the binding of Sp1 to the hypermethylated *Pomc* promoter was significantly reduced. Therefore, we suggest that hypermethylation on the promoter region of the *Pomc* gene can emerge at post-lactation periods and interfere with transcription factor binding, thus blocking the effects of high leptin levels, leading to obesity.

1. Introduction

The regulation of food intake and energy expenditure is maintained by neuropeptides expressed in neurons within nuclei located in the mediobasal hypothalamus

* Corresponding author. Tel.: +972 8 9484411; fax: +972 8 9475075.
E-mail addresses: asafmarco@gmail.com (A. Marco), tatiana.kisliouk@mail.huji.ac.il (T. Kisliouk), weller.aron@gmail.com (A. Weller), meiri@agri.huji.ac.il (N. Meiri).

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(Horvath, 2006; Schwartz et al., 2000). On the one hand, orexigenic neuropeptides, such as neuropeptide Y (NPY) and agouti-related protein (AgRP), increase food intake and body weight. For counter regulation, anorexigenic neuropeptides, such as the post-transcriptional cleavage product of proopiomelanocortin (POMC), alpha-melanocyte-stimulating hormone (alpha-MSH) are expressed in the ARC, mediating satiety and increasing energy expenditure (Myers et al., 2010; Schwartz et al., 2000). The hypothalamic expression of orexigenic and anorexigenic neuropeptides is mainly regulated by peripheral circulating hormones. One of the most important effectors is leptin, an adipose-derived hormone, released proportionally to the levels of body fat stores. Increased levels of leptin and insulin (via ObRb and insulin receptors (IR)) up-regulate hypothalamic expression of anorexigenic neuropeptides, leading to increased energy expenditure and reduced body weight (Cone, 2005). Exogenously administrated leptin induces the expression levels of POMC by a variety of signal transduction mechanisms in normal fed rats (Cowley et al., 2001; Elias et al., 1999). One of these signals is mediated by specificity protein 1 (Sp1) binding to the *Pomc* promoter, an essential step in mediation of the leptin effect (Yang et al., 2009).

Plasma leptin levels are higher in obese subjects, as expected from their higher fat mass compared with lean individuals (Schwartz et al., 2000). Thus, impaired response of the brain to the leptin signal has been shown to result in a persisting dysregulation of food intake and energy homeostasis (Tups, 2009). The inability or reduced efficiency of leptin signaling to affect downstream physiological pathways, found in obesity, is termed 'leptin resistance' (Myers et al., 2008). However, the molecular mechanisms underlying this phenomenon are still unclear. Here we tested, whether epigenetic mechanisms, like CpG DNA methylation, underlie this phenomenon.

In contrast to the DNA sequence, which is determined from conception, the pattern of epigenetic markers may potentially change over the life-span (McGowan and Szyf, 2010; Roth, 2012). It is suggested that different environmental exposures, including a high caloric environment, could affect epigenetic patterns in hypothalamic feeding-control centers and thereby can lead to regulatory system disruptions and obesity. The promoter area of the *Pomc* gene contains a number of CpG dinucleotides that can be either methylated or unmethylated under different weight-gain situations (Plagemann et al., 2009a; Stevens et al., 2010). It is well established that hypermethylation affects transcription factor binding and disrupts its activity (Berger, 2007). Furthermore, studies at the *Pomc* promoter have shown that even hypermethylation upstream of a binding sequence of Sp1 can block its activity (Zhu et al., 2003). In this study we investigated whether such epigenetic programming occur at post-lactation stages as a result of chronic high fat diet consumption. Further, we examined the epigenetic effect on POMC's anorexogenic mechanism focusing on the Sp1 signal transduction pathway, which is essential for the mediation of leptin effects on *Pomc* expression. Sp1 putative binding sites and sites upstream were examined.

2. Materials and methods

2.1. Subjects

Male Wistar outbred rats (supplied by Harlan laboratories LTD, Jerusalem) were raised in the Specific Pathogen Free (SPF) colony of the Gonda Brain Research Center at Bar-Ilan University, Ramat Gan, Israel. In all of the experiments, polycarbonate cages (18.5 cm height, 26.5 cm width, 43 cm length) were used, with stainless steel wire lids and wood shavings as bedding material. In addition, large plastic tubes were present in all of the cages, available for use as a ''house'' or a hiding place for the rats. Standard chow (2018SCF Teklad Global 18% Protein 6% Fat Rodent Diet HARLAN, Madison, USA), or High fat diet (D12492 Research Diets, Inc. Rodent diet with 60% Fat, NJ, USA) and water were freely available. The animals were on a 12:12 h, light:dark cycle, with lights on at 0600 h. Room temperature was maintained at 22 ± 2 °C. Litter size was adjusted on postnatal day (PND) 1 to 8–10. Male rats were raised from PND 21 till PND 90 on either high fat diet (HF group, $N = 16$) or standard rodent chow (C group, $N = 18$). In most cases, two male siblings were selected randomly from a litter and they were allocated to the two groups randomly. In a few cases, only one male pup per litter entered the study. Mean body weight of the pups at weaning was 43.2 (SEM = 1.4) g and 43.6 (SEM = 1.9) g for the C and HF groups, respectively (group difference, NS). Food intake was measured daily and body weight was measured every fifth day. At PND 90 rats were sacrificed; plasma and hypothalamic regions were collected for hormonal, mRNA and DNA methylation analyses (N per group for each analysis is presented in the figure legends). The methylation analysis was performed on a subset of the sample ($N = 5$ C and 6 HF rats) that provided in addition all other measures. The research protocol was approved by the Institutional Animal Care and Use Committee, and it adhered to the guidelines of the Society for Neuroscience. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Tissues and plasma

Trunk blood for leptin and insulin analyses was collected in chilled heparinized vacutainer tubes coated with EDTA. Plasma was stored at −80 °C until assayed. Plasma leptin and insulin levels were assessed using commercial ELISA kits (R&D Systems, Minneapolis, MN and EMD Millipore Corporation, Billerica, MA, USA), according to the manufacturer's instructions. Animals were sacrificed by decapitation (between 1200 h and 1400 h) and brains were removed and immediately frozen on dry ice and stored at −80 °C. Coronal brain sections were cut using a cryostat. The hypothalamic ARC was located (−2.3 to −4.5 mm from Bregma according to Paxinos & Watson's stereotaxic coordinates atlas). Next, a coronal section of 2 mm was cut with a razor and a punch was extracted from the brain, using a truncated needle. For RNA and DNA purification, the left punch from each rat was immediately immersed in RNALater (Ambion, Austin, TX, USA). The right punch was immediately frozen on dry ice for the ChIP and protein analyses.

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