Chapter 2

Induction of brain-region specific forms of obesity by Agouti

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ABSTRACT

Disruption of melanocortin (MC) signalling, such as by ectopic Agouti overexpression, leads to an obesity syndrome with hyperphagia, obesity, and accelerated body weight gain during high fat diet. To investigate where in the brain disruption of MC signalling results in obesity long-term Agouti expression was induced following local injections of recombinant adeno-associated viral particles in selected brain nuclei of adult rats. Agouti expression in the paraventricular nucleus (PVN), a hypothalamic region with a high density of MC receptors, induced acute-onset hyperphagia and rapid weight gain that persisted for at least 6 weeks. In contrast, obesity and hyperphagia developed with a three weeks delay when Agouti was expressed in the dorsal medial hypothalamus (DMH). Agouti expression in the lateral hypothalamus (LH) did not affect food intake and body weight during regular diet, despite the presence of MC receptors in this region. However, during exposure to a high fat diet, animals with Agouti expression in the LH exhibited a marked increase in body weight. Here we show that the LH is important for the protection against diet-induced obesity by controlling caloric intake during consumption of a high fat diet. Taken together, this study provides evidence that different aspects of the Agouti-induced obesity syndrome, such as hyperphagia and diet-responsiveness, are mediated by distinct brain regions and opens challenging opportunities for further understanding of patho-physiological processes in the development of the obesity syndrome.

INTRODUCTION

Genetic studies have shown that the yellow coat colour and the obesity syndrome in rodents with ectopic Agouti overexpression are regulated independently by Agouti antagonism of the melanocyte-stimulating hormone receptor-1 (MC1-R) and of the MC4-R, respectively (81;100;143;304). While Agouti antagonism of the MC1-R in the skin causes a yellow fur by switching eumelanin into phaeomelanin pigment synthesis (144), it is not fully understood where and how Agouti expression generates the obesity syndrome. Since pharmacological blockade or genetic disruption of the MC4-R lead to hyperphagia and obesity (100;123;305), and the MC4-R is expressed in distinct brain areas (76;78;306), it is hypothesised that central Agouti expression accounts for hyperphagia and subsequent body weight gain within the yellow obese syndrome.

Although the melanocortin signaling pathway is clearly involved in body weight control by affecting food intake (81;100) and diet-responsiveness (170), it is poorly understood whether one or different brain sites mediate those MC-effects. Other investigators have used intranuclear injection of melanocortin ligands to study these questions (123;125), however, this approach is limited by the relatively short time of action of these compounds in tissues, which does not allow measurement of obesity development over weeks or months. To study the relationship between MC pathway involvement in the obesity syndrome and the functional anatomy of the MC system we locally interfered with melanocortin receptor signalling using vector directed gene expression technology.

The paraventricular nucleus of the hypothalamus (PVN) contains a high density of MC-4 receptors (MC4-R) that have been proposed to mediate MC-induced changes in food intake. For example, single injections of MC4-R ligands in the PVN alter food intake and body weight in rodents (123;125). Recent studies suggest that the dorsal medial hypothalamus is also involved in the regulation of hyperphagia (307). In addition, based on the outcome of classical lesion, electric stimulation and electro-physiological studies, the lateral hypothalamus (LH) has long been implicated in the regulation of energy metabolism (2;308;309). This idea is corroborated by the recent identification of LH-neurons that express neuropeptides involved in eating behaviour, such as hypocretins/orexins and the melanin-concentrating hormone (MCH) (28;310;311). PVN-, DMH- and LH-neurons express moderate to high levels of MC4-R (76;78;306). As a first step towards understanding where in the brain disruption of MC signalling results in obesity rats with PVN-, DMH- or LH-injections of either rAAV-Agouti or rAAV-EGFP were monitored when exposed to a regular or a high-fat diet.

MATERIAL AND METHODS

Recombinant adeno-associated viral vector production

Construction of pTR-CMVEGFP has been described before (312). Agouti was PCR amplified using as template a plasmid containing the mouse Agouti cDNA (paE65, a kind gift of Roger D. Cone, OHSU) using oligos 5'aagcttgagatctgccgcaccatggatgtcacccg and 5'gaagaagctagctcagcagttggggttg. A (Bgl II and Nhe1 digested) fragment, containing the Agouti cDNA was cloned into Bam HI and Spe I digested pTR-CMV-EGFP (this removes the EGFP cDNA; correct sequence of Agouti was confirmed by sequence analysis) which generated pTR-CMVAgouti. Next, rAAV was generated via a two-component, adenovirus-free packaging system using the helper plasmid pDG (kindly provided by J. Kleinschmidt (313)).

Recombinant AAV particles were produced as described by the method of Hermens et al. (314). Briefly, the vector plasmid pTR-CMVAgouti or pTR-CMV-EGFP and the helper plasmid pDG were cotransfected into human embryonic kidney 293T cells using calcium phosphate precipitation (molar plasmid ratio 1:1). The medium was replaced after 6h by fresh DMEM containing 10% FCS and the cells were incubated for 48h at 37°C and 5% CO_2. Next, the cells were dislodged, harvested and freeze-thawed three times to release the AAV-particles from the cells. Cell debris was removed using low-speed centrifugation. The supernatant was loaded on a Matrex Cellufine sulphate bead column (Amicon, Danvers, MA, USA). After several washings with phosphate-buffered saline PBS), the virus was eluted from the column with PBS containing 1 M NaCL. Next, viral particles were banded on a Iodixanol (Nycomed Pharm, Oslo, Norway) density gradient using ultracentrifugation and fractions af about 300 µl were collected from the bottom of the gradient. To reduce

viscosity of the Iodixanol, rAAV-fractions were diluted 10 times with PBS and reconcentrated on a Centricon-100 concentrator (Amicon). The rAAV stocks contained 2×10^{12} particles/ml for rAAV/Agouti/WPRE and 2×10^{11} particles/ml for rAAV/EGFP/WPRE.

In vitro infection, Western blot analysis and MC receptor activation assay

HEK-293 cells were infected (MOI = 10) with rAAV-Agouti or rAAV-EGFP. 4 days after infection, supernatant was collected. 20 μl Aliquots of the supernatant were diluted 1:1 with tricine sample buffer and separated on a 12% Tris/tricine gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and blocked by incubation for 1 hr at room temperature with 10% non-fat milk in Tris-buffered saline/0.1% Tween 20 (TBST). The membrane was incubated overnight with rabbit anti-Agouti antibody as the primary antibody (1:5,000 dilution), followed by goat-anti-rabbit peroxidase (1:10,000 dilution) (Jackson Immunoresearch Laboratories Inc. West Grove, Pennsylvania, USA) for 60 min after washing with TBST. After washing with TBST, HRP-labeled antibodies were detected by chemiluminescence. Agouti- or EGFP-expressing HEK 293 cells were mixed at a 1:1 ratio with HEK 293 cells co-transfected with 100 ng of a MC4 receptor expression vector (315) and 7 μg of Cre-lacZ (316). Two days following transfection, melanocortin-induced (0.01 – 100 nM MT-II) beta-galactosidase activity was quantified using a colorimetric assay (316). EC_{50} values of MC-induced beta-galactosidase activity in presence of Agouti- or EGFP-producing cells were determined by fitting the data to a sigmoidal curve with variable slope using GraphPad Prism 2.01 for Windows 95/NT (GraphPad Software Inc. San Diego, California, USA).

Animals

Male outbred rats (strain Wistar (U:WU)) (body weights between 240 - 260 grams) were maintained in a 12-h light / 12-h dark cycle (ambient temperature was 21.0 ± 1.0 °C). Food (complete laboratory chow Hope Farms, Woerden, The Netherlands) and water were available ad libitum. After 10 days of baseline recordings (body weight and food intake), rats were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beersse, Belgium; 0.1 ml / 100 gram body weight; i.p.) and Dormicum® (Hoffman-LaRoche, Mijdrecht, The Netherlands; 0.05 ml / 100 g body weight; i.m.). Stereotaxic bilateral injections of rAAV-Agouti and rAAV-EGFP (as a control) were performed in the PVN (-1.8 mm anterior-posterior [AP], 0.3 mm medial-lateral [ML], and -8.6 mm dorsal-ventral [DV] using bregma as a reference for AP and ML co-ordinates and the skull as a reference for DV co-ordinates), DMH (-2.5 mm AP, 0.3 mm ML and 8.4 mm DV), LH (-2.56 mm AP, 1.55 mm ML and 9.32 mm DV) and 0.76 mm anterior of the PVN region (-1.04 mm AP, 0.3 and -0.3 mm ML and -8.6 mm DV). During 5 minutes, 1 μl of virus (2×10^{8} particles) was injected per site. After each injection, the needle remained stationary for an additional 5 min and was then removed.

Following surgery, food intake and body weight were monitored at least every third day for no less than 42 days. Animals were then provided with a high-energy diet (HED) with a high fat content for ten consecutive days (energy content of diets: 3731.9 kcal/kg in regular chow and 4655 kcal/kg in the HED; percentage energy derived from carbohydrate/protein/fat in diets: 63/23/14 in regular chow and 19/19/62 in the HED). After these ten days, all animals were anesthetized with sodium pentobarbital and perfused with saline followed by perfusion with 4% paraformaldehyde (PFA). Brains were post-fixed overnight with 4% PFA and cryoprotected in a 25% sucrose solution (overnight). Brains were than frozen in cold isopentane (-30 °C for 20 seconds). Cryostate sections (20 μm) were used for Agouti immunohistochemistry and for in situ hybridisation.

Agouti antibody staining

Following pre-treatment with 4% fetal calf serum and 0.3% H_2O_2 in PBS, sections were incubated with a rabbit anti-Agouti antibody (1:5000, overnight at 4 ºC). Following a 1 hour incubation with a secondary goat-anti-rabbit-biotinylated antibody (1:100) (Jackson Immunoresearch Laboratories Inc. West Grove, Pennsylvania, USA), section were incubated with ABC (1:500) (Vector Laboratories, Burlingame, California, USA) for 1 hour at room temperature. Sections were then treated with diaminobenzidine (1:100) (Sigma Chemical Co.) in PBS with 30% H_2O_2 in PBS for 10 minutes. All immunohistochemistry steps described above were followed by a 3 times rinse with PBS of at least 5 minutes per rinse. Slides were dehydrated in serial ethanol solutions, cleared with xylene and coverslipped. For histological confirmation of the injection site, Agouti immuno-reactivity in the injection sites was confirmed in every other section throughout the hypothalamus for all animals.

In situ hybridization

Pretreated 20 μm cryostate sections from rat hypothalamus were hybridized with ^{35}S-labeled antisense mRNA probes for AgRP, NPY, MCH, and orexins according to van der Kraan et al. (317). mRNA expression in the arcuate nucleus (for NPY and AgRP) and lateral hypothalamus (for MCH and hypocretins/orexins) was quantified using MCID-M5 (Imaging Research, Ontario, Canada). mRNA levels are expressed in counts per minute (cpm) as calculated from a standard curve of diluted probe mix on the same film as the slides were measured on. From each animal, two measurements per probe were taken in the region of interest (arcuate nucleus and lateral hypothalamus) and subsequently averaged to calculate the mean cpm's per probe for that region.

Statistics

All data are expressed as mean ± S.E.M. Differences in food intake, body weight, feeding efficiency and mRNA expression levels were assessed using one-way and repeated

measure analysis of variance (ANOVA), unless indicated differently in the text. In the presence of a significant main effect, the analysis was followed by Tukey (SPSS for Windows, version 9.0) contrasts ($\alpha=0.05$).

Ethical commission

Animal research and care was approved by the Ethical Commission on Laboratory Animal Experiments of the School of Medicine, University of Utrecht, The Netherlands.
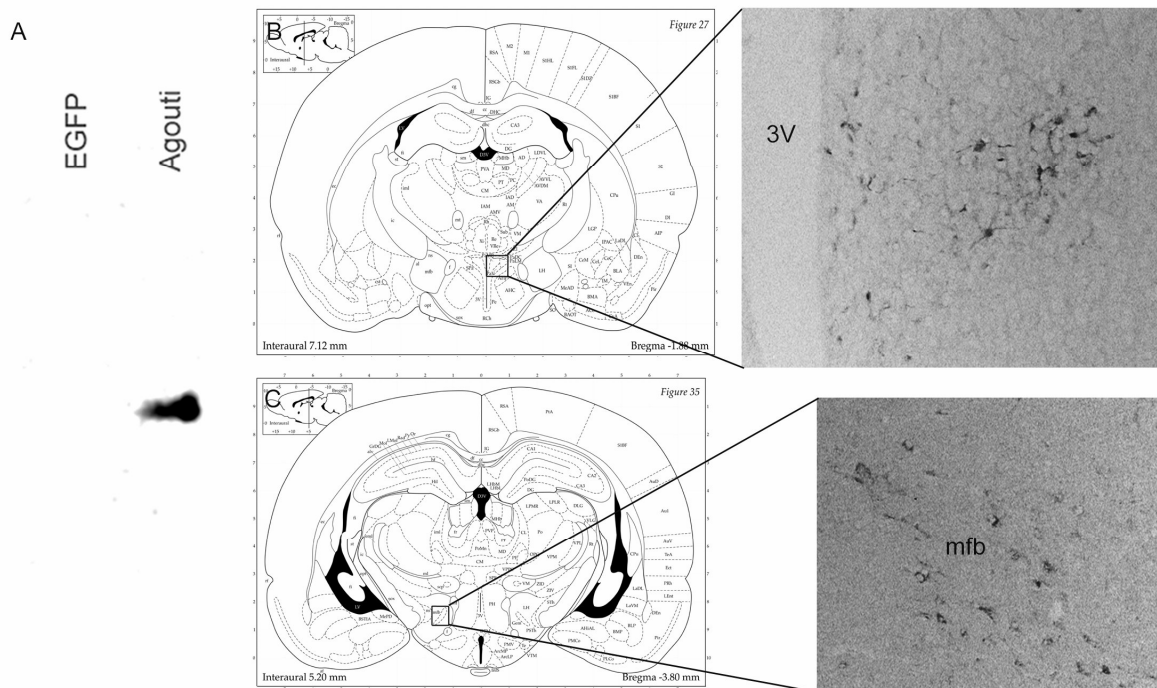
Figure 1: Agouti expression and secretion following infection with the rAAV-Agouti vector. Western blots (A) of media from HEK 293 cells that were infected with the AAV- EGFP-control (left lane) and AAV-Agouti (right lane) vectors were stained with an Agouti-antibody. No Agouti was observed in the media of cells infected with the AAV-EGFP controls (A). Infection by rAAV-Agouti in neurons of the paraventricular nucleus (PVN) of the hypothalamus (B) and lateral hypothalamus (LH) (C) led to local expression of Agouti without staining observed outside of the injected target sites (abbreviations: 3v = third ventricle; mfb = medial forebrain bundle). Regions (B and C) were defined by using Paxinos and Watson (321).
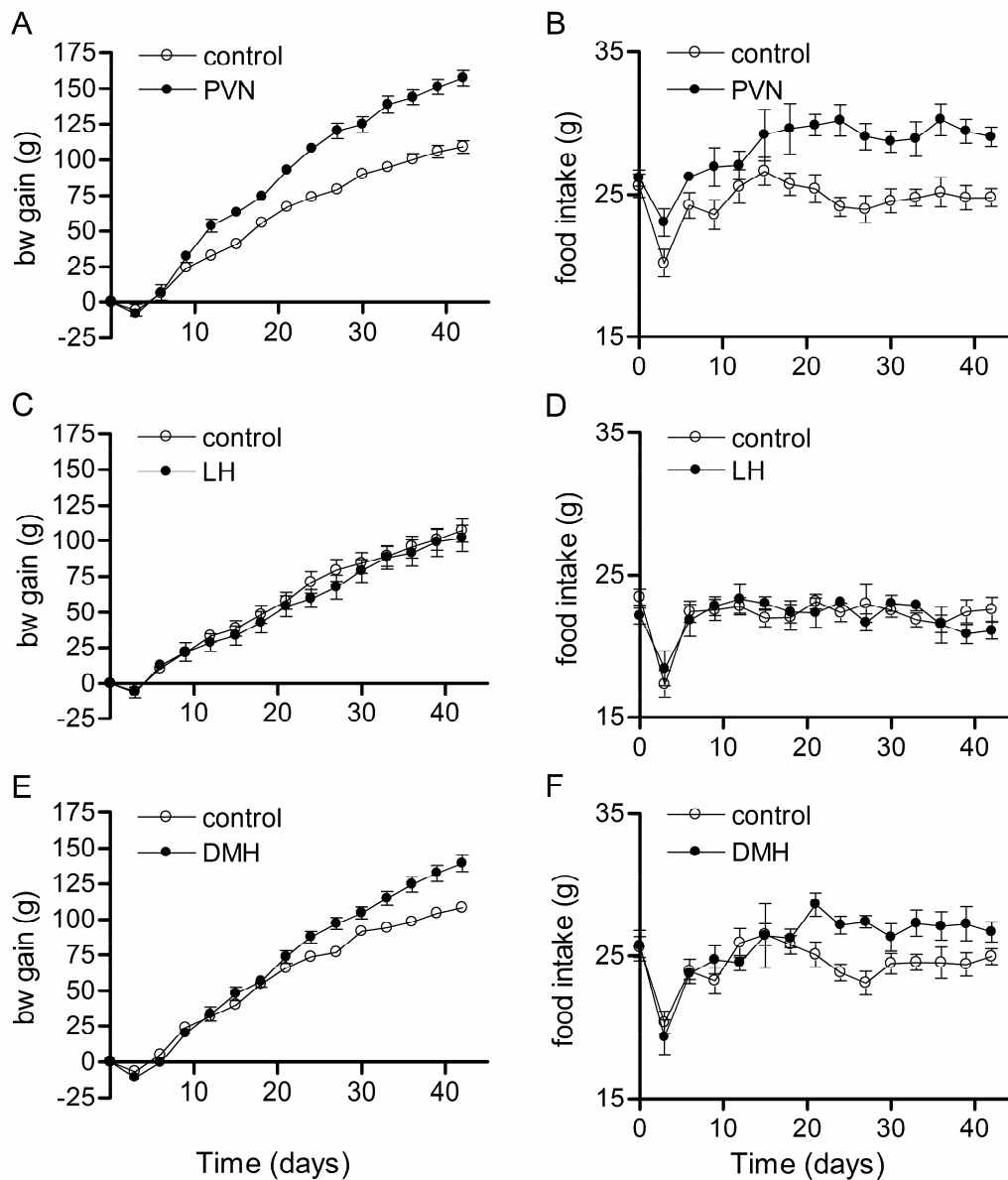
RESULTS

A rAAV-Agouti vector under the control of a CMV-promotor was generated and tested for efficacy to release the Agouti protein upon infection. In contrast to the media of HEK 293 cells infected with the rAAV- enhanced green fluorescent protein (EGFP) control particles, substantial Agouti protein was demonstrated in the media of cells infected with

the rAAV-Agouti particles by western blot analysis (Figure 1A). Thus, Agouti was produced and released from cells infected with rAAV-Agouti viral particles. Injection of 1 μl of the rAAV-Agouti particles (2 x 10^8 particles) in selected brain regions resulted in local Agouti expression (Figures 1B and 1C). It was confirmed that Agouti protein was expressed for at least 60 days with an expression onset as early as three days following injection. No Agouti staining in target sites of these neurons, such as the brain stem, was observed. In order to demonstrate efficacy of Agouti to antagonize MC-induced receptor activation, HEK 293 cells expressing either EGFP or Agouti were mixed with cells transfected with human MC-4 receptor and with Cre-LacZ (as a reporter for receptor stimulation), mimicking rAAV-Agouti infection *in vivo* with cells releasing Agouti in the surrounding of cells that express MC-4 receptors. The EC_{50} value of MC-induced MC-4 receptor stimulated Cre-lacZ in HEK 293 cells was right-shifted from 0.25 nanomolar to 1.1 nanomolar. This indicates that the released Agouti protein functionally antagonises MC-4 receptor signalling.

Expression of the MC4-R antagonist Agouti in the PVN of adult rats increased body weight and food intake within seven days following the injection of rAAV-Agouti as compared to injection of rAAV-EGFP (Figures 2A-B). These elevated levels of food intake and body weight gain remained for over 6 weeks. In this episode, PVN-injected rats ate on average 4 grams per day more than controls, resulting in a 50 % increase in body weight gain (p=0.0001). Histological confirmation of Agouti expression indicated that injections leading to Agouti expression just outside of the PVN-region (missed; n=5) were insufficient to induce the rapid onset hyperphagia, in contrast to animals with confirmed PVN Agouti expression (n=7) (average food intake for the last three days of the 6 weeks of *ad libitum* access to regular chow was 24.8 ± 0.6 g (PVN-EGFP-control), 24.6 ± 0.8 g (missed PVN-Agouti), 29.0 ± 0.7 (PVN-Agouti)). Despite dense levels of MC4-R expression, we show that Agouti expression in the LH, in contrast to that in the PVN, did not affect body weight and food intake when animals were maintained on regular chow (Figures 2C-D).

Unlike the rapid onset of obesity in PVN animals, onset was delayed when Agouti was expressed in the DMH. In the first three weeks following the injection, DMH-injected animals had similar levels of food intake and body weight when compared to controls. Thereafter, however, DMH-injected animals increased food intake and ate on average 2.5 grams per day more than controls. This resulted in a 30% increase in body weight gain (Figures 2E-F; p=0.0001). Since the DMH is located only 0.76 mm posterior from the PVN region involved in acute onset obesity, we investigated whether the delayed onset in DMH animals was a consequence of Agouti leakage to the PVN region. To exclude this possibility, we showed that rAAV-Agouti did not increase food intake and body weight when injected 0,76 mm anterior, instead of posterior, to the PVN region. To also exclude the possibility that the delayed onset of increased food intake and body weight resulted from a later start time

Figure 2: Agouti-expression in the PVN and DMH results in acute and delayed onset long-term hyperphagia-induced obesity, respectively. Body weight gain (A) and food intake (B) (both averaged over three days segments) are increased in animals with Agouti expression in the PVN when compared to EGFP-control PVN-injected animals. During *ad libitum* access to regular chow, body weight gain and food intake were similar in animals with Agouti expression in the LH and EGFP-control LH-injected animals (C and D). Agouti expression in the DMH the onset of increased body weight gain (E) and food intake (F) was delayed by three weeks, in contrast to the acute obesity onset in animals with Agouti expression in the PVN. In the first three days following surgery (days 0-3), a decline in body weight gain and food intake was observed in all animals. Histological verification of Agouti expression was checked for all animals by means of Agouti immuno-reactivity. Animals with Agouti positive cells in the targeted regions (PVN: -1.80 to -2.12 mm from bregma; LH: -2.56 to -3.80 mm from Bregma; DMH: -2.56 to -3.14 mm from Bregma, (region as defined by Paxinos and Watson, the rat brain in stereotaxic coordinates, 4th edition, 1998) were used for the analysis and resulted in the shown behavioural data-set for these confirmed groups (n=6-7 per group). * indicates p<0.0001.

of expression of AAV-constructs in the DMH when compared to the PVN, we confirmed that Agouti was expressed in the DMH as early as 3 days following the injection.

High fat food is known to contribute to the development of obesity. Since Agouti expression in the LH did not contribute to food intake and body weight during regular chow following regular diet, all animals were maintained on a high-energy diet with a high fat content for 10 consecutive days to test diet responsiveness. This high fat diet resulted in increased body weight gain in LH-Agouti animals when compared to LH-EGFP controls (Figure 3A). Usually, this diet results in a suppression of daily food intake because of the higher caloric density of food (318). However, animals with Agouti expression in the LH, had a far less pronounced suppression of food intake when they were switched to a high fat diet when compared to LH-EGFP-control, DMH, and PVN-Agouti injected animals. Indeed, actual comparison of caloric intake during regular and high fat diet in LH-Agouti and LH-EGFP control animals revealed that LH-Agouti animals had an increased caloric intake during the high fat diet (Figure 3B).

Local long-term Agouti expression in the PVN, DMH and LH differentially induced hyperphagia and accelerated body weight gain without apparent changes in central neuropeptide systems that are known to stimulate food intake. We found that both AgRP and NPY mRNA levels in arcuate nucleus neurons, as well as MCH and orexin mRNA expression in the lateral hypothalamus were not affected following long-term Agouti expression in the PVN, DMH or LH (Figure 4).
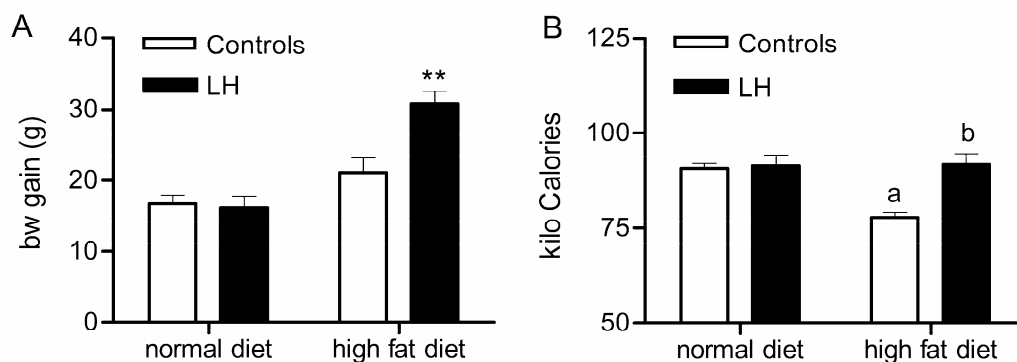
Figure 3: Agouti expression in the LH induced diet-induced obesity. While LH-EGFP control and LH-Agouti injected animals had similar levels of body weight gain during a normal diet (body weight gain during the last ten days of the normal diet), body weight gain significantly increased in animals with Agouti expression in the LH during ten days of access to a high fat diet (as compared to body weight on the day prior to the high fat diet) (A). This increased body weight gain in LH-Agouti injected animals related to increased caloric intake in LH-Agouti animals during the high fat diet (B) (** indicates different from all other conditions (p<0.0001); a indicates different from LH-EGFP during normal diet (P=0.006); b indicates different from LH-EGFP during high fat diet (P=0.003)).
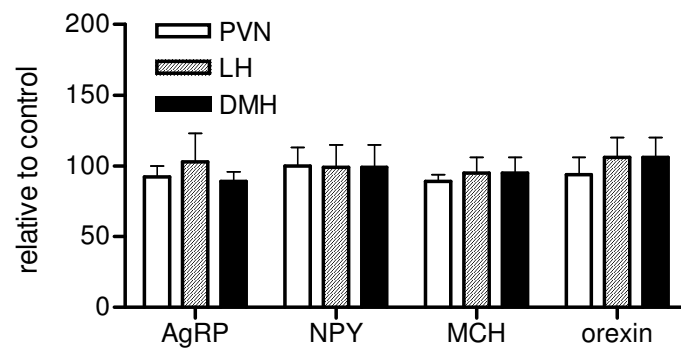
Figure 4: Hypothalamic mRNA expression levels of the orexigenic neuropeptides AgRP and NPY in the arcuate nucleus, as well as MCH and orexins in the lateral hypothalamus. Following long-term local expression of Agouti in the PVN, DMH and LH, no difference in mRNA expression levels were observed when compared to EGFP-controls (in brains of rats from Figures 2 and 3). mRNA expression levels are expressed as a percentage from the EGFP-controls. Since region specific EGFP-controls were not different in gene expression levels, their expression levels were pooled for the analysis.

DISCUSSION

Our results show that Agouti expression in the PVN induced a rapid onset of hyperphagia and body weight gain. This was not unexpected, since a single local injection of a MC agonist stimulates food intake (123;125). Surprisingly, Agouti expression in the DMH caused delayed onset of increased food intake and body weight, whereas LH-Agouti expression did not affect body weight on a regular diet, but resulted in diet-induced obesity. Therefore, these data show that MC signalling in different brain regions contributes to different characteristics of the obesity syndrome and that the development of these characteristics, such as hyperphagia and diet-induced obesity, are under distinct neuro-anatomical control in the brain. These findings open opportunities for further research aimed at how these different brain regions control different aspects of energy homeostasis. Furthermore, the approach taken is readily available to investigate whether disruption of MC signalling in other brain regions implicated in regulation of food intake, such as the dorsal motor nucleus of the vagus nerve, also induce characteristics of the obesity syndrome.

Here we show that Agouti expression in the PVN and DMH both stimulates food intake and body weight, however, the timing of hyperphagia onset is significantly delayed in the DMH when compared to the PVN. Since these regions are closely located to each other, the DMH effects of Agouti may be explained by leakage of Agouti from the DMH to the PVN resulting in a delayed onset of the PVN-induced hyperphagia. However, an acute or a delayed onset of hyperphagia and body weight increase were not found in animals with Agouti expression just outside the PVN (missed PVN-injections) or 0.76 mm anterior to PVN (the DMH is located 0.76 mm posterior to PVN). Therefore, these data make it unlikely that

the delayed onset of hyperphagia following DMH-Agouti expression was caused by leakage of Agouti from the DMH to the PVN and indicate that disruption of MC signalling in the DMH does not result in immediate increased food intake and body weight, such as observed following AAV-Agouti injection in the PVN. Further studies can now focus on the mechanisms underlying this late onset obesity and the role of the DMH therein.

The present data show that inhibition of MC signalling in the LH selectively accelerates the development of obesity on a high fat diet by affecting caloric intake. LH-Agouti injected animals had similar caloric intake and body weight gain on regular chow. However, Agouti expression in the LH resulted in an increased caloric intake and subsequent body weight gain on a high fat diet when compared to LH-EGFP controls. Therefore, our data provide evidence that the LH is an important brain region for the protection against diet-induced obesity rather than for the induction of hyperphagia (as shown in the PVN). The increased caloric intake following inhibition of MC signalling in the LH may result from impaired satiety signalling during intake of high fat food. In addition, recent studies suggest that the LH region integrates information about energy status and reward (319). Further behavioural and anatomical studies are needed to demonstrate how energy intake, energy expenditure and possible reward mechanisms are integrated in the LH.

To further characterize the different mechanisms underlying the brain region specific forms of Agouti-induced obesity, mRNA gene expression levels of known orexigenic neuropeptides (NPY, AgRP, MCH, and Orexins) were measured in several brain regions. Although local and long-term Agouti expression in the PVN, DMH or LH had marked and different effects on body weight and food intake, no obvious changes in mRNA expression levels of these known orexigenic neuropeptide systems were observed. One interpretation of these results is that brain region-specific disruption of MC-R signalling by Agouti leading to hyperphagia and accelerated weight gain on a high fat diet is independent of regulation of the hypothalamic orexigenic neuropeptides NPY, AgRP, MCH, and orexins. This idea is consistent with previous data showing that blockade of MC-R signalling, while causing hyperphagia and obesity, did not lead to alterations in expression levels of a number of hypothalamic neuropeptides including, for example, NPY expression in the arcuate nucleus (43;305). We cannot rule out, however, that induction of Agouti expression did transiently alter expression of, at least, some of these neuropeptide systems allowing these animals to become obese. Therefore, a more extensive analysis of time-dependent changes in these neuropeptide systems and other metabolic parameters may be required to further study physiological changes related to the observed brain region specific forms of obesity induced by Agouti.

Taken together, these findings suggest that a single neuropeptide system, such as the MC system, regulates different adaptive behavioural and physiological strategies to alter an

organism's energy balance towards a certain direction (e.g., energy conservation) in distinct brain nuclei. The brain homolog for Agouti, AgRP is expressed in the arcuate nucleus of the hypothalamus and innervates a wide variety of brain regions, such as the PVN, DMH and LH. These arcuate nucleus neurons respond to sudden changes in peripheral molecules, such as leptin, glucose and insulin, that provide information about the energy status of an organism. Starvation, for example, leads to a drop in leptin levels (320) and subsequent increase in AgRP gene expression (7). This will result in a simultaneous activation of distinct brain regions that, as indicated by the present study, regulate brain region specific aspects of physiological processes, each contributing separately to energy conservation.