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Published in:
The Journal of Neuroscience

DOI:
10.1523/JNEUROSCI.3442-04.2004

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2004

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Induction of Brain Region-Specific Forms of Obesity by Agouti

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Disruption of melanocortin (MC) signaling, 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 signaling results in obesity, long-term Agouti expression was induced after local injections of recombinant adeno-associated viral particles in selected brain nuclei of adult rats. Agouti expression in the paraventricular nucleus, 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 3 week delay when Agouti was expressed in the dorsal medial hypothalamus. 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. 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 pathophysiological processes in the development of the obesity syndrome.

Key words: melanocortin; obesity; lateral hypothalamus; dorsal medial hypothalamus; paraventricular nucleus; high-fat diet

Introduction

Genetic studies have shown that the yellow coat color 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 (Duhl et al., 1994; Lu et al., 1994; Fan et al., 1997; Huszar et al., 1997). Although Agouti antagonism of the MC1-R in the skin causes a yellow fur by switching eumelanin into phaeomelanin pigment synthesis (Ollmann et al., 1998), it is not fully understood where and how Agouti expression generates the obesity syndrome. Because pharmacological blockade or genetic disruption of the MC4-R lead to hyperphagia and obesity (Huszar et al., 1997; Kim et al., 2000; Adage et al., 2001) and the MC4-R is expressed in distinct brain areas (Gantz et al., 1993; Mountjoy et al., 1994; Kishi et al., 2003), it is hypothesized 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 (Fan et al., 1997; Huszar et al., 1997) and diet responsiveness (Butler et al., 2001), it is poorly understood whether one or different brain sites mediate these MC effects. Other investigators have used intranuclear injection of melanocortin ligands to study these questions (Kim et al., 2000; Wirth et al., 2001); 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 signaling using vector directed gene expression technology.

The paraventricular nucleus of the hypothalamus (PVN) contains a high density of MC4-Rs 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 (Kim et al., 2000; Wirth et al., 2001). Recent studies suggest that the dorsal medial hypothalamus (DMH) is also involved in the regulation of hyperphagia (Chen et al., 2004). In addition, based on the outcome of classical lesion, electric stimulation, and electrophysiological studies, the lateral hypothalamus (LH) has long been implicated in the regulation of energy metabolism (Anand and Brobeck, 1951; Delgado and Anand, 1953; Katafuchi et al., 1985). This idea is corroborated by the recent identification of LH neurons that express neuropep-
tides involved in eating behavior, such as hypocretins/orexins and the melanin-concentrating hormone (MCH) (Zamir et al., 1986; de Lecea et al., 1998; Sakurai et al., 1998). PVN, DMH, and LH neurons express moderate to high levels of MC4-R (Gantz et al., 1993; Mountjoy et al., 1994; Kishi et al., 2003). As a first step toward understanding where in the brain disruption of MC signaling results in obesity, rats with PVN, DMH, or LH injections of either recombinant adeno-associated virus (rAAV)-Agouti or rAAV-enhanced green fluorescent protein (EGFP) were monitored when exposed to a regular or a high-fat diet.

Materials and Methods

Recombinant adeno-associated viral vector production. Construction of pTR-cytomegalovirus (CMV)-EGFP has been described previously (Eaton et al., 2002). Agouti was PCR amplified using as template a plasmid containing the mouse Agouti cDNA (paE65; a kind gift from Roger D. Cone, Oregon Health and Science University, Portland, OR) using oligos 5’-aagcttgagatctgccgcaccatggatgtcacccg and 5’-gaagaagctgtagcagttggggttg. A (BglII and Nhel digested) fragment containing the Agouti cDNA was cloned into BamHI and SpeI digested pTR-CMV-EGFP (this removes the EGFP cDNA; correct sequence of Agouti was confirmed by sequence analysis), which generated pTR-CMV-Agouti. pTR-CMV-Agouti was generated via a two-component, adenovirus-free packaging system using the helper plasmid pDG (kindly provided by J. Kleinschmidt, Deutsches Krebsforschungszentrum, Heidelberg, Germany (Grimm et al., 1998)).

Recombinant AAV particles were produced by the method described previously by Hermens et al. (1999). Briefly, the vector plasmid pTR-CMV-Agouti or pTR-CMV-EGFP and the helper plasmid pDG were cotransfected into human embryonic kidney (HEK) 293T cells using a two-component, adenovirus-free packaging system (multiplicity of infection, 10) (Jackson ImmunoResearch WestGrove, PA) for 60 min incubation. All immunohistochemistry steps described above were followed by a three times rinse with PBS of at least 5 min per rinse. Slides were dehydrated in serial ethanol solutions, then incubated with diaminobenzidine (1:100) (Sigma, St. Louis, MO) in PBS containing 30% H2O2 in PBS for 10 min. All immunohistochemistry steps described above were followed by a three times rinse with PBS of at least 5 min per rinse. Slides were dehydrated in serial ethanol solutions, cleared with xylene, and coverslipped. For histological confirmation of the injection site, Agouti immunoreactivity in the injection sites was confirmed in every other section throughout the hypothalamus for all animals.

In situ hybridization. Pretreated 20 μm cryostat sections from rat hypothalamus were hybridized with 35S-labeled antisense mRNA probes for Agouti-related protein (AgRP), NPY, MCH, and orexins according to van der Kraan et al. (1998). mRNA expression in the arcuate nucleus (for MCH and hypocretins/orexins) was quantified using MCID-M5 (Imaging Research, St. Catharines, Ontario, Canada). mRNA levels are expressed in counts per minute as calculated from a standard curve of diluted probe mix on the same film on which the slides were measured. 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 counts per minute per probe for that region.

Results

An rAAV-Agouti vector under the control of a CMV promotor was generated and tested for efficacy to release the Agouti protein after infection. In contrast to the media of HEK 293 cells infected...
with the rAAV-EGFP control particles, substantial Agouti protein was demonstrated in the media of cells infected with the rAAV-Agouti particles by Western blot analysis (Fig. 1A). Thus, Agouti was produced and released from cells infected with rAAV-Agouti viral particles. Injection of 1 μl of the rAAV-Agouti particles \(2 \times 10^8\) particles in selected brain regions resulted in local Agouti expression (Fig. 1B, C). It was confirmed that Agouti protein was expressed for at least 60 d with an expression onset as early as 3 d after injection. No Agouti staining in target sites of these neurons, such as the brainstem, was observed. 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 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 to 1.1 nm. This indicates that the released Agouti protein functionally antagonizes MC-4 receptor signaling.

Expression of the MC4-R antagonist Agouti in the PVN of adult rats increased body weight and food intake within 7 d after the injection of rAAV-Agouti compared with injection of rAAV-EGFP (Fig. 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 gm/d 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 3 d of the 6 weeks of \textit{ad libitum} access to regular chow was 24.8 ± 0.6 gm (PVN-EGFP control), 24.6 ± 0.8 gm (missed PVN-Agouti), and 29.0 ± 0.7 gm (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 (Fig. 2C, D).

Unlike the rapid onset of obesity in PVN animals, onset was delayed when Agouti was expressed in the DMH. In the first 3 weeks after the injection, DMH-injected animals had similar levels of food intake and body weight when compared with controls. Thereafter, however, DMH-injected animals increased food intake and ate on average 2.5 gm/d more than controls. This resulted in a 30% increase in body weight gain \(p = 0.0001\) (Fig. 2E, F). Because 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 of expression of AAV constructs in the DMH when compared with the PVN, we confirmed that Agouti was expressed in the DMH as early as 3 d after the injection.

High-fat food is known to contribute to the development of obesity. Because Agouti expression in the LH did not contribute to food intake and body weight during regular chow, 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 with LH-EGFP controls (Fig. 3A). Usually, this diet results in a suppression of daily food intake because of the higher caloric density of food (Adolph, 1947). 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 with 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 (Fig. 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 hypothalamic region, were not affected after long-term Agouti expression in the PVN, DMH, or LH (Fig. 4).

Discussion

Our results show that Agouti expression in the PVN induced a rapid onset of hyperphagia and body weight gain. This was not unexpected, because a single local injection of a MC agonist stimulates food intake (Kim et al., 2000; Wirth et al., 2001). Surpris-
ingly, 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 signaling 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 neuroanatomical control in the brain. These findings open opportunities for additional 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 signaling 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 tim-

**Figure 2.** Agouti expression in the PVN and DMH results in acute and delayed onset long-term hyperphagia-induced obesity, respectively. Body weight (bw) gain (A) and food intake (B) (both averaged over 3 d segments) are increased in animals with Agouti expression in the PVN when compared with 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, D). In animals with Agouti expression in the DMH, the onset of increased body weight gain (E) and food intake (F) was delayed by 3 weeks.

**Figure 3.** Agouti expression in the LH induced diet-induced obesity. Whereas LH-EGFP control and LH-Agouti injected animals had similar levels of body weight (bw) gain during a normal diet (body weight gain during the last 10 d of the normal diet), body weight gain significantly increased in animals with Agouti expression in the LH during 10 d of access to a high-fat diet (compared with body weight on the day before 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). **p < 0.0001, different from all other conditions; **p = 0.006, different from LH-EGFP during normal diet; **p = 0.003, different from LH-EGFP during high-fat diet.

**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. After long-term local expression of Agouti in the PVN, DMH, and LH, no difference in mRNA expression levels were observed when compared with EGFP controls (in brains of rats from Figs. 2, 3). mRNA expression levels are expressed as a percentage from the EGFP controls. Because region-specific EGFP controls were not different in gene expression levels, their expression levels were pooled for the analysis.

In contrast to the acute obesity onset in animals with Agouti expression in the PVN. In the first 3 d after 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 immunoreactivity. 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 using the atlas by Paxinos and Watson (1998)) were used for the analysis and resulted in the shown behavioral data set for these confirmed groups (n = 6 – 7 per group).
ing of hyperphagia onset is significantly delayed in the DMH when compared with the PVN. Because 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 after DMH-Agouti expression was caused by leakage of Agouti from the DMH to the PVN and indicate that disruption of MC signaling in the DMH does not result in immediate increased food intake and body weight, such as observed after AAV-Agouti injection in the PVN. Additional 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 signaling 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 with 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 after inhibition of MC signaling in the LH may result from impaired satiety signaling during intake of high-fat food. In addition, recent studies suggest that the LH region integrates information about energy status and reward (Fulton et al., 2000). Additional behavioral 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 signaling 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 signaling, although 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 (Kesterson et al., 1997; Adage et al., 2001). 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.

Together, these findings suggest that a single neuropeptide system, such as the MC system, regulates different adaptive behavioral and physiological strategies to alter an organism’s energy balance toward 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 (Ahima et al., 1996) and subsequent increase in AgRP gene expression (Hahn et al., 1998). 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.

References
Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berke meier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Camp-


