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REVIEW

The MC4 receptor and control of appetite

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Mutations in the human melanocortin (MC)4 receptor have been associated with obesity, which underscores the relevance of this receptor as a drug target to treat obesity. Infusion of MC4R agonists decreases food intake, whereas inhibition of MC receptor activity by infusion of an MC receptor antagonist or with the inverse agonist AgRP results in increased food intake. This review addresses the role of the MC system in different aspects of feeding behaviour. MC4R activity affects meal size and meal choice, but not meal frequency, and the type of diet affects the efficacy of MC4R agonists to reduce food intake. The central sites involved in the different aspects of feeding behaviour that are affected by MC4R signalling are being unravelled. The paraventricular nucleus plays an important role in food intake per se, whereas MC signalling in the lateral hypothalamus is associated with the response to a high fat diet. MC4R signalling in the brainstem has been shown to affect meal size. Further genetic, behavioural and brain-region specific studies need to clarify how the MC4R agonists affect feeding behaviour in order to determine which obese individuals would benefit most from treatment with these drugs. Application of MCR agonists in humans has already revealed side effects, such as penile erections, which may complicate introduction of these drugs in the treatment of obesity.
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Keywords: melanocortins; AgRP; POMC; obesity; feeding behaviour; MC4 receptor

Abbreviations: AgRP, agouti-related protein; CCK, cholecystokinin; CRH, corticotropin-releasing hormone; CTA, conditioned taste aversion; DMH, dorsomedial hypothalamus; DVC, dorsovagal complex; i.c.v., intracerebroventricular; LH, lateral hypothalamus; LHSS, lateral hypothalamic self stimulation; MC, melanocortin; MCH, melanin concentrating hormone; MSH, melanocyte-stimulating hormone; MTII, melanotan-II; NTS, nucleus of the tractus solitarius; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; TRH, thyrotropin-releasing hormone; VMH, ventromedial hypothalamus

Introduction

In order to maintain stable body weight, energy intake (by ingestion) and energy expenditure (by exercise, basal metabolism and thermogenesis) need to be balanced. The constant availability of highly palatable foods and the lack of exercise strongly contribute to the development of obesity (Woods *et al*., 2004). Drugs that effectively reduce body weight are urgently needed to fight the obesity epidemic (Boss and Bergenhem, 2006). The unravelling of the genetic defect underlying obesity in the *ob/ob* mouse, namely the absence of the leptin gene, was key towards identification of neural pathways and neuropeptides that control body weight (Zhang *et al*., 1994; Friedman and Halaas, 1998). Leptin is an adipose tissue-derived hormone that is released into the circulation proportional to increased energy stores in fat. Leptin stimulates neural circuits that decrease food intake and increase energy expenditure. Both humans and rodents with mutations in the *leptin* gene or leptin receptor gene are obese (Zhang *et al*., 1994; Chen *et al*., 1996; Lee *et al*., 1996; Montague *et al*., 1997; Clement *et al*., 1998; Barsh *et al*., 2000).

The arcuate nucleus of the hypothalamus is an important relay centre for leptin's effects. The hypothalamic arcuate nucleus integrates and distributes peripheral information from hormonal and neural signals that reflect metabolic status further into the brain (Figure 1). Within the arcuate nucleus, neurons containing melanocortins (MCs), products of the *pro-opiomelanocortin* (*POMC*) gene, are activated by leptin (Schwartz *et al*., 1997). These neurons provide one of the systems via which the leptin signal is propagated further into the brain. Fasting results in loss of adipose tissue and low leptin levels, which causes diminished activation of POMC neurons, whereas overfeeding (high leptin levels) results in a stimulation of POMC neurons (Mizuno *et al*., 1998; Hagan *et al*., 1999). Thus, POMC neurons are stimulated during a positive energy balance,

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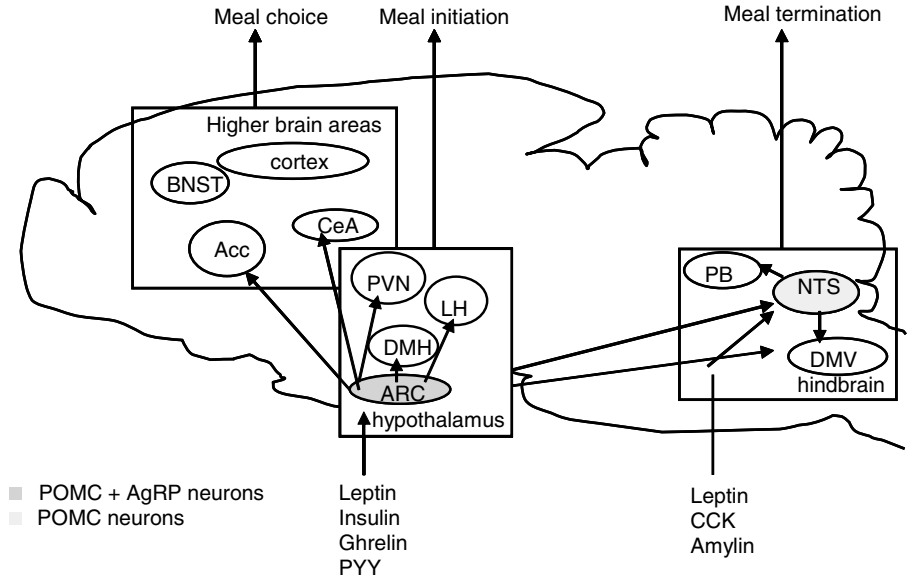

Figure 1 Simplified scheme of the role of melanocortins in food intake. BNST, bed nucleus of the stria terminalis; Acc, accumbens; CeA, Amygdala; PVN, paraventricular nucleus of the hypothalamus; LH, lateral hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; ARC, arcuate nucleus; PB, parabrachial nucleus; NTS, nucleus of the tractus solitarius; DMV, dorsomotor nucleus of the vagus.

and increased plasma leptin levels contribute to this stimulation.

The activity of the MC system is not only regulated by the endogenous MC receptor agonists, α-melanocyte-stimulating hormone (α-MSH), β-MSH and γ-MSH, which are all derived from the POMC precursor, but also by agouti-related protein (AgRP; Table 1). *AgRP* is also expressed in the arcuate nucleus but in a different subset of neurons than those expressing POMC. In contrast to POMC neurons, AgRP neurons are inhibited by leptin and activated during negative energy balance. Although often described as a competitive antagonist, AgRP acts in fact as an inverse agonist on constitutively active MC3 and MC4 receptors (Haskell-Luevano and Monck, 2001; Nijenhuis *et al*., 2001), the main brain MC receptors. The unique presence of an endogenous agonist and an inverse agonist acting at the same receptor system implicates a tight regulation of MC function in the brain. Thus, during a negative energy balance, AgRP neurons are activated and AgRP acts to suppress MC receptor activity.

Although the MC system modulates energy expenditure and insulin sensitivity (Cone, 2005), this review focuses on the role of the MC system in regulation of energy intake. After summarizing the evidence that the MC4 receptor is a promising drug target for the treatment of obesity, it will be discussed how and via which neural circuits the MC system affects energy intake.

Mutations in the MC system: lessons from the MC4 receptor knockout mouse

Spontaneous as well as genetically introduced variations in genes constituting the MC system demonstrate the importance of this system in body weight homeostasis. The first gene belonging to the MC system that was deleted in mice was the *MC4 receptor*. Overall, disruption of the *MC4 receptor* in mice results in an obese phenotype, underscoring the potential of the MC4 receptor as drug target for obesity (Huszar *et al*., 1997; Chen *et al*., 2000b).

MC4 receptor$^{-/-}$ display maturity onset obesity characterized by hyperphagia, increased adiposity, increased longitudinal growth, normal lean body mass, hyperinsulinaemia and hyperleptinaemia. *MC4 receptor*$^{+/-}$ show an intermediate phenotype with respect to body weight and food intake, suggesting a gene dosage effect (Huszar *et al*., 1997; Chen *et al*., 2000b). When female *MC4 receptor*$^{-/-}$ mice are pair-fed to controls, their body weight are intermediate between wild-type and non-pair-fed mice, suggesting that inhibition of MC4 receptor activity also affects energy balance independent from food intake (Ste Marie *et al*., 2000). Furthermore, non-pair-fed *MC4* knockout mice consume less oxygen per gram body weight than wild types (Ste Marie *et al*., 2000). Interestingly, *MC4* knockout mice are already heavier than wild types before hyperphagia is significantly increased (Ste Marie *et al*., 2000). Although body temperature of *MC4 receptor*$^{-/-}$ is normal, locomotor activity of male *MC4 receptor*$^{-/-}$ is reduced in the dark phase (Marsh *et al*., 1999a; Ste Marie *et al*., 2000). Thus, obesity of *MC4 receptor*$^{-/-}$ mice is due to both hyperphagia and reduced energy expenditure.

Peripheral as well as central leptin administration does not reduce food intake in obese *MC4 receptor*$^{-/-}$, whereas young non-obese *MC4 receptor*$^{-/-}$ have an attenuated response to leptin (Marsh *et al*., 1999a). The MC4 receptor does not seem to be involved in the orexigenic or anorexigenic effects of neuropeptide Y (NPY), peptide YY (PYY), orexins and urocortin, as the response to these neuropeptides on food intake is normal in *MC4 receptor*$^{-/-}$ (Marsh *et al*., 1999a; Halatchev *et al*., 2004). On the other hand, cholecystokinin (CCK), a gut-derived satiety factor has an attenuated response (inhibiting food intake) in *MC4*$^{-/-}$ (Fan *et al*.,

Table 1 Overview of MCR endogenous ligands, locations and functions

MC receptor subtype	*Endogenous ligand (decreasing affinity)*	*Endogenous antagonist*	*Central location*	*Peripheral location*	*Central function*	*Peripheral function*
MC1	αMSH = βMSH = ACTH > γMSH	Agouti	Periaquaductal grey	Melanocyte, pituitary, placenta, testis, corpus luteum, macrophages, monocytes, neutrophils, endothelium, glioma cells, astrocytes, fibroblasts, keratinocytes, Th cells, natural killer (NK) cells		Pigmentation, anti-inflammatory
MC2	ACTH	Agouti		Adrenals, murine adipocytes, skin, Th cells, NK cells, monocytes, granulocytes		Glucocorticoid production, stress-induced lipolysis
MC3	γMSH = αMSH = βMSH = ACTH	AgRP	Brainstem, hypothalamus, thalamus, septum	Placenta, gut, heart, thymus, murine macrophages, Th cells, NK cells, monocytes, granulocytes	Energy homeostasis, anti-inflammatory	Pro-inflammatory cytokine release
MC4	αMSH = βMSH = ACTH > γMSH	AgRP, agouti	Brainstem, hypothalamus, thalamus, striatum, septum, cortex, hippocampus, limbic system, spinal cord		Body weight regulation, grooming, pain processing, sexual behaviour	Penile erections
MC5	αMSH > βMSH = ACTH > γMSH	Agouti	Cortex, cerebellum, striatum, midbrain, pons, medulla, olfactory bulb	Pituitary, skin, adrenals, fat cells, smooth and skeletal muscle, bone marrow, spleen, lymph nodes, thymus, gonadals, uterus, lung, liver, stomach, oesophagus, kidney, mammary glands, exocrine glands, Th cells, NK cells, monocytes, granulocytes		Natriuresis, sebum secretion, preputial lipogenesis
Ref	(Schioth *et al*., 1996; Adan and Gispen, 1997)	(Ollmann *et al*., 1997; Yang *et al*., 1997)	(Roselli-Rehfuss *et al*., 1993; Griffon *et al*., 1994; Mountjoy *et al*., 1994; Fathi *et al*., 1995; Xia *et al*., 1995)	(Getting *et al*., 1999; Wikberg, 1999; Wikberg *et al*., 2000; Andersen *et al*., 2005)	(Gispen *et al*., 1975; Hirsch *et al*., 1984; Huszar *et al*., 1997; Chen *et al*., 2000a; Muceniece *et al*., 2006)	(Thody *et al*., 1976; Leiba *et al*., 1990; Robbins *et al*., 1993; Bhardwaj *et al*., 1997; Lipton and Catania, 1998; Boston, 1999; Gantz and Fong, 2003)

Abbreviations: ACTH, adrenocoticotropic hormone; AgRP, agouti-related protein; MC, melanocortin; MCR, melanocortin receptor; MSH, melanocyte-stimulating hormone.

Table 2 Overview of the effects of administration of neuropeptides on food intake and body weight in wild-type and MC4 receptor$^{-/-}$ animals

Neuropeptide	*Wild type*		*MC4*$^{-/-}$		*Reference*
	Food intake	*Body weight*	*Food intake*	*Body weight*	
Orexigenic					
AgRP	↑	↑	↑/=	↑/=	(Marsh *et al*., 1999b; Small *et al*., 2001; Fekete *et al*., 2004)
β-Endorphin	↑				(Tsujii and Bray, 1989)
Galanin	↑/=	=			(Smith *et al*., 1994)
Ghrelin	↑	↑	=		(Wren *et al*., 2001; Shaw *et al*., 2005)
MCH	↑	↑			(Gomori *et al*., 2003)
NPY	↑	↑	↑		(Stanley *et al*., 1986; Marsh *et al*., 1999a)
Orexin	↑/=	=	=		(Haynes *et al*., 1999; Marsh *et al*., 1999a)
Anorexigenic					
αMSH	↓	↓	=[a]	=[a]	(Marsh *et al*., 1999a; McMinn *et al*., 2000)
Amylin	↓	↓			(Rushing *et al*., 2000)
βMSH	↓				(Kask *et al*., 2000)
CART	↓	↓			(Rohner-Jeanrenaud *et al*., 2002)
CCK	↓	↓/=	=		(Schick *et al*., 1988; Fan *et al*., 2004)
CRH	↓	↓	↓	↓	(Krahn *et al*., 1990; Marsh *et al*., 1999a)
Insulin	↓	↓			(Brief and Davis, 1984)
Leptin	↓	↓	=	=	(Halaas *et al*., 1997; Marsh *et al*., 1999a)
Neurotensin	↓	=			(Levine *et al*., 1983)
PYY	↓	↓	↓		(Batterham *et al*., 2002; Halatchev *et al*., 2004)
TRH	↓				(Vijayan and McCann, 1977)
Urocortin	↓		↓	↓	(Marsh *et al*., 1999a; Reyes *et al*., 2001)

↑, increased compared to saline administration; ↓, decreased compared to saline administration; =, normal compared to saline administration.
[a]Effect after MTII administration, empty fields, no data available.
Abbreviations: AgRP, agouti-related protein; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; CRH, corticotropin-releasing hormone; MCH, melanin concentrating hormone; MSH, melanocyte-stimulating hormone; NPY, neuropeptide Y; PYY, peptide YY; TRH, thyrotropin-releasing hormone.

2004), which suggests that intact MC4 receptor signalling is implicated in CCK's effect on satiety (Table 2).

Mutations in other genes of the MC system

Genetic deletion of the *MC3 receptor*, the other main MC receptor in the brain (Table 1), also resulted in an energy balance phenotype. *MC3 receptor*$^{-/-}$ display adiposity, while not being hyperphagic, and are hypoactive (Butler *et al*., 2000; Chen *et al*., 2000a). Besides being expressed in multiple other brain sites, the *MC3 receptor* is expressed in the arcuate nucleus on POMC neurons (Jegou *et al*., 2000). This suggests that the MC3 receptor plays a role as autoreceptor, normally suppressing the activity of POMC neurons. If the MC3 receptor normally limits POMC neuronal activity, then lack of the *MC3 receptor* on POMC neurons would increase its activity, as a negative feedback signal would be disrupted. Subsequent release of MCs would increase MC4 receptor activity and this may contribute to the relative hypophagia observed in *MC3* knockout mice. The increased leptin levels owing to adiposity would further contribute to this hypophagia. This might explain why *MC3 receptor*$^{-/-}$ are not hyperphagic, but hypophagic, despite being fat and hyperleptinemic. Recently, it was demonstrated that peripheral injection of an MC3 receptor selective agonist increased food intake (Marks *et al*., 2006). This is consistent with an autoinhibitory role of the MC3 receptor expressed on POMC neurons in the arcuate nucleus.

AgRP knockout mice do not have an obvious phenotype (Qian *et al*., 2002). As *AgRP* is co-expressed with *NPY* in the arcuate nucleus, *AgRP* knockouts were crossed with NPY knockouts. Surprisingly, double knockouts were not lean, suggesting that NPY and AgRP do not play an essential role in feeding behaviour and body weight homeostasis (Qian *et al*., 2002). However, upon closer examinations (by comparing younger and older mice), it appeared that older *AgRP* knockouts display increased locomotor activity, increased thyroid hormone levels and increased energy expenditure, which results in a late-onset lean phenotype (Wortley *et al*., 2005). This is in agreement with results obtained by suppression of *AgRP* expression in the adult stage by RNA interference (RNAi), resulting in increased metabolic rate contributing to decreased body weight (Makimura *et al*., 2002). *AgRP* expression can also be reduced by damaging AgRP neurons. By expressing the human diphtheria toxin receptor specifically in AgRP neurons, AgRP neurons can be destroyed selectively once diphtheria toxin is administered at chosen moments during development. When the toxin is administered at early stages of development, animals have a normal phenotype. However, administration of diphteria toxin in the adult stage resulted in a lean phenotype (Luquet *et al*., 2005). Using another strategy, Xu *et al*. (2005) demonstrated that ablation of AgRP neurons results in decreased fat mass. These data indicate that interpretation of results obtained from knockout mice is complicated by compensations for lack of components of the MC system during development.

POMC knockout mice are also obese, which is explained by the lack of endogenous MCs. Interestingly, *POMC*$^{-/-}$ have adrenal insufficiency and, subsequently, very low corticosterone levels and are hypersensitive to the metabolic effects of glucocorticoids (Yaswen *et al*., 1999). Recently, Low and co-workers demonstrated that when *POMC* was re-expressed

in pituitary gland of *POMC* knockouts using a transgenic approach, an obesity phenotype was obtained, which was more pronounced than in *POMC* knockouts (Smart *et al*., 2006). This may be explained by the presence of restored hypothalamo–pituitary–adrenal (HPA) function in these *POMC* knockout mice with transgenic expression of *POMC* in pituitary gland, as compared to overall *POMC* knockout mice.

Feeding behaviour in humans with mutations in the MC system

Mutations in the *MC4 receptor* are a relatively common cause of severe childhood obesity (Farooqi *et al*., 2000, 2003; Vaisse *et al*., 2000, Mackenzie, 2006). The carrier prevalence for *MC4* mutations in a juvenile-onset obese population has been noted to be around 2.5% with a highest prevalence of 6% among severe obese children (Vaisse *et al*., 2000; Farooqi *et al*., 2003; Larsen *et al*., 2005; Hinney *et al*., 2006). Humans with *MC4* mutations show a more or less similar phenotype as has been described for mice with mutations in the *MC4 receptor* gene. Those people show clear hyperphagia, hyperinsulinaemia, increased fat mass, lean body mass, bone mineral density and linear growth rate, with no changes in cortisol levels, gonadotropin, thyroid and sexsteroid levels (Farooqi *et al*., 2000, 2003). In contrast to *MC4 receptor*$^{-/-}$, hyperphagia and hyperinsulinaemia tends to subside with age in human subjects. Similar to the *MC4* knockout mice, the phenotype in heterozygote carriers is intermediate in comparison to homozygote carriers (Farooqi *et al*., 2003). The exhibited hyperphagia observed upon a test meal is less severe than that observed in people with a leptin deficiency (Farooqi and O'Rahilly, 2005). The severity of MC4 receptor dysfunction seen in assays *in vitro* can predict the amount of food ingested at a test meal by the subject harbouring that particular mutation and correlates with the onset and severity of the obese phenotype (Farooqi *et al*., 2003; Lubrano-Berthelier *et al*., 2006). At least 90 different *MC4 receptor* mutations have been associated with obesity. Many of these mutations cause retention of the mutated receptors intracellularly, resulting in loss of agonist response (Lubrano-Berthelier *et al*., 2003; Nijenhuis *et al*., 2003). In other mutations, a selective loss of agonist affinity and response were reported (Yeo *et al*., 2003). Even mutations in the *human MC4 receptor* that only decrease its constitutive activity, without a loss in agonist responsiveness, have been associated with obesity (Srinivasan *et al*., 2004). This underscores the *in vivo* relevance of constitutive MC4 receptor activity for normal weight maintenance, and provides a means for AgRP to act beyond α-MSH as inverse agonist. Indeed, even subtle changes in the activity of the MC system, such as in human heterozygous carriers of mutations in the *MC4 receptor* gene, result in an increased susceptibility to develop obesity (Santini *et al*., 2004).

Besides mutations in the *MC4 receptor*, mutations in the *POMC* gene have been described which lead to severe obesity in humans and to the same phenotype as observed in obese people with an *MC4 receptor mutation* (Krude *et al*., 1998, 2003; Challis *et al*., 2002). Until recently, the effect of mutations in the *POMC* gene have been attributed to a malfunctioning of α-MSH on the MC4 receptor, as α-MSH is the peptide important for signalling via the MC4 receptor in rodents. However, two recent papers indicate that also mutations in *POMC* leading to a disruption of β-MSH lead to an obese phenotype similar to that of *MC4 receptor* mutations, supporting a role for β-MSH in the control of energy balance in humans (Biebermann *et al*., 2006; Lee *et al*., 2006).

In contrast to the *MC4 receptor* and *POMC* genes, mutations in the human *AgRP* gene have not been identified so far. However, there are some reports on polymorphisms in the *AgRP* gene that need to be replicated. Two of such polymorphisms in the human *AgRP* gene appear to influence energy intake. The 67Thr-allele of *AgRP* is associated with reduced energy intake from fat and enhanced energy intake from carbohydrates in white subjects (Loos *et al*., 2005). Another allele, characterized by a −38 C/T in the *AgRP* promoter region is associated with protein intake in black subjects. Homozygotes of the T allele show reduced protein intake as compared to carriers of the C allele. The TT genotype is associated with a low promoter activity and low affinity for transcription factors and was previously found to be protective of obesity (Mayfield *et al*., 2001; Argyropoulos *et al*., 2003). These data are consistent with data described above regarding the phenotype of mice carrying deletions of the *AgRP* gene and ablation of AgRP neurons: a lean phenotype was induced.

Pharmacological studies using melanocortin receptor ligands

The discovery and application of specific agonists and antagonists for MC receptors contributed strongly to unravel the role of the MC system in energy balance. Central administration of non-specific MC receptor agonists such as α-MSH and melanotan-II (MTII) results in decreased food intake and body weight (Fan *et al*., 1997). *MC receptor* knockout mice provide an interesting model to determine which (an)orexigenic signals are dependent on which MC receptor in their effect on appetite. While MTII administration in wild-type control mice inhibits fasting-induced refeeding, this is not observed in *MC4 receptor*$^{-/-}$ (Marsh *et al*., 1999a). Furthermore, MTII also does not inhibit nocturnal feeding or 24 h feeding in freely feeding *MC4 receptor*$^{-/-}$ (Marsh *et al*., 1999a; Chen *et al*., 2000b) as it does in wild-type mice. As MTII still inhibits food intake in *MC3 knockout* mice (Chen *et al*., 2000a), the *MC3 receptor* is probably not involved in reduction of food intake induced by MC receptor agonists. This indicates that the central MC pathways that inhibit appetite act through the MC4 receptor.

Centrally administered AgRP, however, does seem to induce hyperphagia in *MC4 receptor*$^{-/-}$. Although the increase in food intake is smaller than in wild-type controls, and not always significant, this suggests that unlike the anorectic effects of MTII, the orexigenic effects of AgRP are not exclusively mediated via the MC4 receptor, but may also involve the MC3 receptor (Marsh *et al*., 1999b; Fekete *et al*., 2004).

MTII activates similar neuronal circuits as leptin and is able to reduce adiposity in leptin-deficient *ob/ob* mice (Bluher *et al*., 2004), which further underscores that MCs act downstream of leptin. MTII also counteracts starvation-induced hyperphagia, suggesting that reduction of MC receptor activity is part of the physiological response to starvation (Fan *et al*., 1997).

Application of the MC 3/4 receptor antagonist SHU9119 results in voluntary overfeeding, obesity and increased plasma leptin levels (Fan *et al*., 1997; Adage *et al*., 2001). Consistent with data obtained in MC4$^{-/-}$ mice, SHU9119 increases body adiposity and plasma leptin levels when rats are pair-fed to controls, suggesting that metabolic effects of reduced brain MC activity are also independent of food intake. A lower body temperature and reduced locomotor activity contribute to these effects (Adage *et al*., 2001). Moreover, MTII is not able to increase metabolic rate in MC4 receptor$^{-/-}$, whereas it does in wild-type littermates, suggesting that the MC4 receptor is indeed necessary for the regulation of metabolism (Chen *et al*., 2000b).

A low dose of SHU9119 prevents compensatory reduction in food intake after involuntary overfeeding (Hagan *et al*., 1999). This illustrates the physiological relevance of activation of the MC system during a positive energy balance. The inverse agonist AgRP is a potent stimulant of food intake and is implicated in the maintenance of feeding rather than the initiation of feeding (Wirth and Giraudo, 2000). Therefore, AgRP is most effective when injected in the natural feeding period or following food deprivation. A single central administration of AgRP increases food intake for at least 24 h (up to 7 days) and blocks α-MSH/MTII-induced hypophagia (Rossi *et al*., 1998; Hagan *et al*., 2000; Wirth and Giraudo, 2000). Chronic treatment of rats with central AgRP increases body weight gain by enhancing food intake and reducing energy expenditure (Small *et al*., 2001, 2003). Taken together, pharmacological evidence suggests that the central MC system affects energy intake as well as energy expenditure and underscores the physiological involvement of the MC system in energy balance.

Effects of melanocortinergic signalling on feeding behaviour

As described above, the central MC system is clearly involved in regulation of feeding behaviour. The question remains, however, which specific biological processes related to appetite control are regulated by MC signalling?

In order to survive, it is essential for an animal to search for food, remember food sources, prepare for consumption, ingest sufficient amounts of food and digest foods efficiently. The decision to eat is controlled by endogenous drives (hunger and satiety), and also by environmental cues such as availability of palatable or novel foods and predator exposure when searching for food (Blundell and Gillett, 2001). Furthermore, different factors involved in food intake influence each other. For example, meal termination is controlled by internal and external cues (Kral, 2006). Meal size is influenced by extent of negative energy balance preceding the meal as well as meal composition (Strubbe and Woods, 2004). Even when a high degree of satiety is achieved, the availability of a highly palatable food may overrule this and increase meal size (Erlanson-Albertsson, 2005). Both, anatomical and pharmacological evidence suggests that satiety, hunger and rewarding aspects associated with feeding are regulated in different anatomical sites, many of which contain MC receptors (Berthoud, 2004). To answer the question of how the MC system affects feeding behaviour, pharmacological and genetic interference studies have been combined with behavioural paradigms that address these different processes.

For example, it was found that similar to leptin, MTII reduces food intake by affecting meal size and duration as shown by meal pattern analysis studies, whereas meal frequency and inter-meal interval were unaffected (Williams *et al*., 2002; Zheng *et al*., 2005). Oppositely, SHU9119 increases food intake by selectively increasing meal size (Zheng *et al*., 2005). These data suggest that the MC system is involved in meal termination rather than meal initiation. This is consistent with the demonstration that MTII is less effective in suppressing food intake when rats expect a meal (Benoit *et al*., 2003) which underscores the lack of MC effects on affecting meal initiation. Other studies by Benoit *et al*. (2003) showed that the efficacy of MC receptor agonists appears attenuated during scheduled feeding, when rats have learned to consume large amounts of food in a short period of time, suggesting that the MC system is not involved in anticipation to food.

Data from analysis of MC4$^{-/-}$ mice do not always fit with these pharmacological data. In a paradigm where mice have to press a lever to get a meal, MC4 receptor$^{-/-}$ are not hyperphagic and lose body weight, while control animals show normal food intake and body weight gain. Meal size as well as frequency is normal in MC4 receptor$^{-/-}$ in this paradigm, suggesting that a functional MC4 receptor is not required for the normal regulation of meal patterns (Vaughan *et al*., 2005). However, data about meal patterns during hyperphagia and in a normal environment are necessary to clarify this. Furthermore, in MC4$^{-/-}$ mice, compensatory adaptation may mask a physiological role of the MC system in determining meal size.

Ligands that suppress food intake may do so because they induce a state of illness. This can be tested by pairing ligand infusion with a flavour, and subsequent measurement of the intake of the flavoured food in the absence of ligand infusion, as in conditioned taste aversion (CTA) tests. Although it has been reported that MTII, the mixed MC3/4 receptor agonist, induced CTA, more selective MC4 receptor agonists do not induce CTA, suggesting that the MC4 receptor is the candidate MC receptor for development of agonists to suppress energy intake (Butler, 2006). Thus, the MC system affects meal size, but not meal initiation, meal frequency or anticipation and a reduction of food intake by activation of the MC4 receptor is not caused by inducing nausea.

Effects of melanocortins on different diets

Relevant to the evaluation of MC receptor agonists as potential drugs to reduce food intake in obesity, is whether

the efficacy of MC receptor agonists depends on the type of diet and whether MC receptor agonists are still effective in obese subjects.

When MC receptor agonists are infused over days to weeks in rats, the efficacy to suppress food intake is high in the first days but fades away over time (McMinn *et al*., 2000; Jonsson *et al*., 2002). Rats that are fed a high-fat diet for several weeks also show an attenuated response to MTII (Benoit *et al*., 2003; Clegg *et al*., 2003). It was recently shown that chronic MTII treatment still reduces food intake in rats fed a high-fat diet as well as in food-deprived rats. MTII was, however, less effective in reducing food intake and body weight in rats with a lower body fat mass (Seeley *et al*., 2005). This indicates that activity of the MC system is related to the defended level of body adiposity, suggesting that the main function of the MC system is regulating body adiposity rather than food intake.

Interestingly, when the high-fat diet is low in carbohydrates (mimicking the 'Atkins' diet), MTII sensitivity is maintained, although the level of adiposity on this diet is increased as compared to controls (Kinzig *et al*., 2005). It is important to note that not all laboratories that investigated the effects of MCs have used the same diet, which complicates direct comparisons. Further studies need to clarify whether the type of (high fat) diet affects the responsiveness of the MC system.

Another aspect of different diets is that these not only differ in caloric density but also in palatability. MC receptors are expressed in brain centres that relay information on taste and palatability, such as the amygdala, nucleus of the tractus solitarius and parabrachial nucleus. This provides an anatomical basis for an effect of MCs on food choice. As MCs may affect taste processing, this might contribute to an overall effect on food intake. There are several reports on the role of the MC system in preference for certain foods. MTII specifically reduces intake of fat (but not of protein or carbohydrates) on a three-choice macronutrient diet (Samama *et al*., 2003) and the inverse agonist AgRP enhances the intake of specifically high-fat diets in rats (Hagan *et al*., 2001). In addition, obese mice with ectopic overexpression of *Agouti* (which mimics the action of AgRP) have enhanced preference for fat meals (Koegler *et al*., 1999).

Also, experiments using *MC4*$^{-/-}$ mice indicate an involvement of the MC system in fat preference. When exposed to a high-fat diet, *MC4 receptor*$^{-/-}$ display an increased caloric intake, which is, unlike in control animals, not normalized after 48 h. Together with an enlarged feed efficiency, this results in an even more increased body weight gain. Additionally, whereas normal animals increase their oxygen consumption on a high-fat diet, this effect is absent in *MC4 receptor*$^{-/-}$. This indicates that the MC4 receptor is required for a normal metabolic and behavioural response to increased dietary fat. Thus, *MC4*$^{-/-}$ have a deficit in the normal response (reduced intake) to a high-fat diet, which may, besides a reduced metabolic response to high-fat diet, be explained by a deficit in sensing foods with an increased caloric density or by increased liking of fat foods. When given the choice between a high-fat, high-protein and high-carbohydrate diet, wild-type animals treated with MTII reduce specifically the intake of the high-fat diet, whereas the intake of high protein and high carbohydrate derived calories remains unchanged. This effect is absent in *MC4 receptor*$^{-/-}$, suggesting that the MC4 receptor is necessary for the MTII-induced reduction of fat intake (Samama *et al*., 2003).

Taken together, accumulating evidence in rodents, but also in humans carrying mutations in the MC system, indicate that reduction of MC receptor activity is associated with preference for fat meals. However, there are long-term effects of different diets, with the ratio of fat to protein and carbohydrates in diets as important factors affecting the sensitivity for MC receptor agonists. It might be that the level of adiposity (which is increased by high-fat diets) sets the sensitivity of the brain MC system. Future studies need to clarify whether treatment with MC4 receptor agonists are able to shift this adiposity set point.

The sites in the brain where MC receptor signalling affects energy intake

The *MC4 receptor* is expressed in several sites in the brain, including hypothalamus, forebrain and hindbrain, that have been implicated in energy balance (Mountjoy *et al*., 1994; Kishi *et al*., 2003). This suggests that a large variety of processes and anatomical sites involved in energy balance are modified by MC4 receptors. Studies investigating the site of action of (an)orexigenic effects of MC receptor ligands within the hypothalamus and amygdala identified the paraventricular (PVN) and dorsomedial (DMH) hypothalamus and medial preoptic (MPO) area as primary sites (Figure 1) (Kim *et al*., 2000).

The PVN receives multiple projections from various areas in the brain and its central location makes it an integrator of signals regulating food intake and body weight. The PVN receives input from the ARC where neuropeptides of the MC system are produced. Local injection of the MC4 receptor agonists MTII or α-MSH into the PVN results in a reduction of food intake in mice as well as in rats. Interestingly, the effects of MCs on food intake are only observed when feeding is stimulated, for instance by the onset of the dark period, by fasting or by central NPY injection (Giraudo *et al*., 1998; Cowley *et al*., 1999; Wirth *et al*., 2001), which underscores an effect on meal termination rather than meal initiation. MC4 receptor antagonists such as AgRP, SHU9119 and HS014 stimulate feeding when administered directly into the PVN (Giraudo *et al*., 1998; Cowley *et al*., 1999; Kask and Schioth, 2000; Wirth and Giraudo, 2000). Again, these antagonists are merely effective when meal initiation is triggered, suggesting that signalling via the MC4 receptor in the PVN is implicated in meal duration rather than meal initiation.

A subpopulation of corticotropin-releasing hormone (CRH) neurons in the PVN contains MC4 receptors, and MTII induces a rapid induction of *CRH* gene transcription in the PVN (Lu *et al*., 2003). The CRH receptor antagonist α-helical-CRH(9–41) partly antagonizes the MTII-food-suppressing effect when injected intracerebroventricularly (i.c.v.), which suggest that CRH acts as a downstream mediator of MC signalling and contributes to the mechanisms by which the central MC system controls feeding (Lu *et al*., 2003).

Thyrotropin-releasing hormone (TRH)-expressing neurons in the PVN also have MC4 receptors, and MC signalling increases *TRH* expression (Harris *et al*., 2001). It is not clear to what extent activation of TRH signalling contributes to the effect of MCs to suppress food intake.

As mentioned above, a second hypothalamic site that shows involvement of the MC system in appetite is the DMH. The DMH plays an important role in relaying information to neural pathways mediating neuroendocrine, autonomic and behavioural responses to stress. [norleucine^4; D-phenylalanine^7]-α-MSH and AgRP administration into the DMH result in hypophagia or hyperphagia, respectively (Kim *et al*., 2000; Wirth and Giraudo, 2001). Furthermore, AgRP administration in the DMH increases sucrose intake, whereas it does not affect the intake of an isocaloric product as corn starch, suggesting an influence of the palatability of a diet (Wirth and Giraudo, 2001).

Also, brainstem *MC4 receptors* are involved in feeding behaviour. *MC4 receptors* are expressed in the dorsal motor nucleus of the vagus nerve and in the nucleus of the tractus solitarius, both these brain centres are important for autonomic control. Grill *et al*. (1998) found that fourth ventricle administration of the MC4 receptor ligands MTII and SHU9119 has comparable effects on food intake as administration to the lateral ventricle, indicating that the caudal brainstem is contributing to the effects of the MC system on appetite. More specifically, Williams *et al*. (2000) injected MTII directly into the dorsal vagal complex (DVC) and this reduced food intake and body weight, whereas SHU9119 stimulates food intake, suggesting that brainstem effects on appetite are mediated by the DVC (Williams *et al*., 2000). Administration of SHU9119 in the third or fourth ventricle revealed that the CCK-mediated inhibition of food intake is affected by MC4 receptor activity in the brainstem but not in the hypothalamus (Fan *et al*., 2004). CCK-sensitive neurons in the nucleus of the tractus solitarius (NTS) have been implicated in mediating the effect of MCs to reduce meal size (Sutton *et al*., 2005).

Two other sites expressing *MC receptors* are the central amygdala (CeA) and the lateral hypothalamus (LH). The CeA is an important relay centre for taste perception. Whereas effects on food intake are absent after administration of the MC4 receptor agonist NDP-MSH into the CeA, the antagonists AgRP or HS014 do increase food intake, although to a lesser extent than injection into the PVN, DMH or DVC (Kask and Schioth, 2000; Kim *et al*., 2000). The LH connects the hypothalamus with the mesolimbic reward system and electrical stimulation of the LH induces food intake in satiated rats. The LH contains two types of neurons expressing orexigenic neuropeptides, namely melanin concentrating hormone (MCH) and orexin neurons. AgRP-increased food intake is associated with increased c-fos expression in orexin, but not MCH-containing neurons in the LH (Zheng *et al*., 2002). However, AGRP administered i.c.v. into rats augmented MCH but not orexin gene expression (Hanada *et al*., 2000). It remains to be determined whether MCH and/or orexin are implicated as downstream effectors of the MC system. MC4 receptor agonists or antagonists injected into the LH, arcuate nucleus, ventromedial hypothalamus and nucleus accumbens have little or no effect on food intake (Kask and Schioth, 2000; Kim *et al*., 2000). Taken together, multiple MC receptor-expressing sites in the brain have been implicated in regulating food intake with the PVN, DMH and DVC/NTS as the most sensitive sites. Further studies need to clarify which aspect of appetite is affected precisely when MC receptor activity in these brain sites is modulated.

Long-term disturbance of melanocortinergic signalling in specific brain sites

Most pharmacological studies show short-term effects on MC signalling and do not study the effects on body weight regulation. Moreover, pharmacological interventions are limited by the relatively short time of action of these ligands, which does not allow measurement of obesity development over weeks or months. By using mice that carry a lox-stop-lox *MC4 receptor* allele, which can be reactivated by *Cre recombinase* expression, Balthasar *et al*. (2005) recently demonstrated that in mice carrying a loss of function allele of the MC4 receptor, local re-expression of MC4 receptors in the PVN and a subpopulation of amygdala neurons normalizes excessive food intake and reduce body weight, but does not reduce energy expenditure (Balthasar *et al*., 2005). By means of a different, but complementary approach, Kas *et al*. (2004) demonstrated that inhibition of MC receptor signalling in the rat PVN by injecting an adeno-associated virus (AAV) expressing *Agouti* resulted in increased food intake and body weight (Kas *et al*., 2004). Thus, by locally rescuing the obese phenotype in a knockout mouse (Balthasar *et al*., 2005) and by a brain region-specific induction of an obese phenotype in a normally developed rat (Kas *et al*., 2004), these studies provide matching evidence for a role of MC signalling in the PVN for regulating food intake. These observations are consistent with studies showing that acute injections of MC ligands in the PVN affect food intake (Kim *et al*., 2000; Wirth *et al*., 2001).

Chronic inhibition of MC signalling through *Agouti* expression in the LH in rats (Kas *et al*., 2004) does not induce obesity on normal chow. However, it was discovered before that in MC4 receptor$^{-/-}$ mice, obesity is strongly increased by ongoing exposure to a high-fat diet (Butler *et al*., 2001). When exposed to a high-fat diet, LH AAV-*agouti*-injected rats develop an obesity phenotype, suggesting that MC signalling in the LH is involved in a normal response to high-fat diets (Kas *et al*., 2004). Thus, different aspects of MC regulation of energy balance, such as food intake, energy expenditure and coping with high-fat diets, are regulated by distinct nuclei in the brain expressing *MC receptors*.

A promising new development is to use viral vectors for delivery of short hairpin (sh) RNAs that knock down gene expression. A lentiviral system has been build now, which allows inducible micro-RNA-based shRNA expression under control of PolII promoters (Stegmeier *et al*., 2005). Certainly, this will contribute to further increase timing and cell-type specificity of gene knockdown. We have successfully generated transgenic mice, expressing an shRNA targeting the *MC4 receptor*. The transgenic *MC4 receptor* knockdown animals showed a similar phenotype as described for the

MC4 receptor$^{-/-}$. They had a 1.3-fold increase in body weight in comparison to their non-transgenic littermates and control lentiviral transgenic mice. This phenotype could be enhanced on a high-fat diet, as has been described for *MC4 receptor*$^{-/-}$ before (De Krom *et al*., unpublished observations).

The melanocortin 4 receptor as drug target

The MC4 receptor is an attractive candidate drug target to treat obesity, as it not only affects several aspects of feeding behaviour as discussed in this review, but activation of the MC system also increases insulin sensitivity and energy expenditure, part of which effect is independent of food intake (Banno *et al*., 2004; Heijboer *et al*., 2005). Several pharmaceutical industries are active in the development of MC4 receptor-specific drugs (Tian *et al*., 2005; Bakshi *et al*., 2006; Nicholson *et al*., 2006). It has proven difficult to design selective MC4 receptor agonists, in particular those that completely lack affinity for the MC3 receptor (Holder and Haskell-Luevano, 2004; Todorovic and Haskell-Luevano, 2005). However, mixed MC3/4 agonism might provide a therapeutic advantage, as reduced MC3 receptor activity (as in MC3$^{-/-}$ mice) has been associated with increased adipogenesis. Early rodent and human studies revealed some side effects of MC receptor agonists. Administration of MC4 receptor agonists is associated with penile erections as well as flushing, which has resulted in a new application area for these drugs: erectile dysfunction (Van der Ploeg *et al*., 2002; Diamond *et al*., 2004; Wessells *et al*., 2005). Preclinical studies have identified roles of the MC system in blood pressure regulation (MC receptor agonists have a depressor effect most probably via the NTS in brainstem) (Versteeg *et al*., 1998), in the inflammatory responses (where agonists limit thermogenic responses to pyrogens) (Catania *et al*., 2004) and in pain processing (where antagonists suppress pain sensation) (Vrinten *et al*., 2001). With the development of MC4 receptor agonists in the treatment of obesity, it should be carefully monitored whether these other MC effects provide unwanted side effects.

Concluding remarks

Interference within the central MC system (e.g. at the level of *AgRP*, *POMC*, *MC3* or of *MC4 receptors*) revealed a wide variety of energy balance phenotypes. Hyperphagia and obesity are observed in both mice and humans with mutations in MC system. Reduction of MC receptor activity is associated with pushing the energy balance towards positive. The MC system affects multiple factors affecting energy balance, such as meal size, food choice and energy expenditure, which are controlled in different brain sites expressing MC receptors. The PVN plays a major role in MC4 receptor-mediated hyperphagia, whereas interactions between hypothalamic (e.g., LH) and mesolimbic signalling may play a role in the normal response to high-fat diets. The role of the MC system in feeding behaviour needs to be unravelled further, in order to select those groups of obese individuals that may benefit from treatment with MC4 receptor agonists. Careful analysis of feeding behaviour in humans treated with MC4 receptor agonists (when they become available for clinical studies) provides an interesting approach to achieve this.

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Conflict of interest

The authors state no conflict of interest.

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