Chapter 1

General introduction

Part of this chapter is adapted from Adan *et.al.,* Br.J.Pharmacol. 2006;149:815

GENERAL INTRODUCTION

NEURAL CIRCUITS INVOLVED IN ENERGY BALANCE

In most situations energy homeostasis is regulated tightly, resulting in a relatively constant body weight even when daily food intake is variable. However, in modern lifestyle with an environment where plenty of (energy dense) food is available and there is less need to exercise, this homeostatic regulation is overruled by non-homeostatic systems which account for the hedonic aspects of feeding behaviour, resulting in an increase in the prevalence of obesity.

It has become evident by pharmacological and anatomical studies that the basic aspects associated with food intake, as hunger, satiety and reward, are regulated in different brain areas, although these different sites are highly interconnected. The hypothalamus is suggested to be the main site for long-term regulation of energy homeostasis, while circuits in the caudal brainstem are involved in acute feeding responses and autonomic outflow. While these areas control homeostasis, non-homeostatic feeding is influenced by corticolimbic structures, which comprise a circuit influenced by cognitive and environmental factors (reviewed in (1)) (Fig. 1).

Hypothalamus

Already since the earliest lesion studies in the 1950s an important role for the hypothalamus in energy homeostasis was found when a model of feeding and satiety centers in the hypothalamus was proposed (2-4). Since then, it has become clear that the hypothalamic regulation of food intake is not as simple as that, but that it involves a complete network of integrated pathways.

In the hypothalamus signals about the metabolic state of the periphery are further integrated in the brain. Peripheral signals, such as the adiposity signals leptin and insulin, signal to the brain via the arcuate nucleus (Arc) of the hypothalamus, which is connected to many hypothalamic regions where both energy intake and energy expenditure are modified (Fig 2).

Arcuate nucleus

The blood brain barrier in the vicinity of the Arc is reduced, making the neurons in the Arc more sensitive to peripheral signals (5).

Two important groups of neurons are located in the Arc; one population produces the orexigenic neuropeptides Agouti-related protein (AgRP) and neuropeptide Y (NPY), the other co-expresses the anorexigenic neuropeptides proopiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) (6;7). The neurons in the Arc respond to leptin and insulin, which circulate in blood at levels that are proportional to body fat (8;9),

but in an opposite manner. Leptin injection in the Arc stimulates POMC/CART neurons and inhibits NPY/AgRP expression and thereby decreases food intake (10-13). Similarly, NPY and AgRP expression is increased in circumstances of a negative energy balance, for instance after fasting, or in animals with a leptin deficiency (7;10;14), while POMC and CART neurons are inhibited in situations when leptin levels are low (13;15).

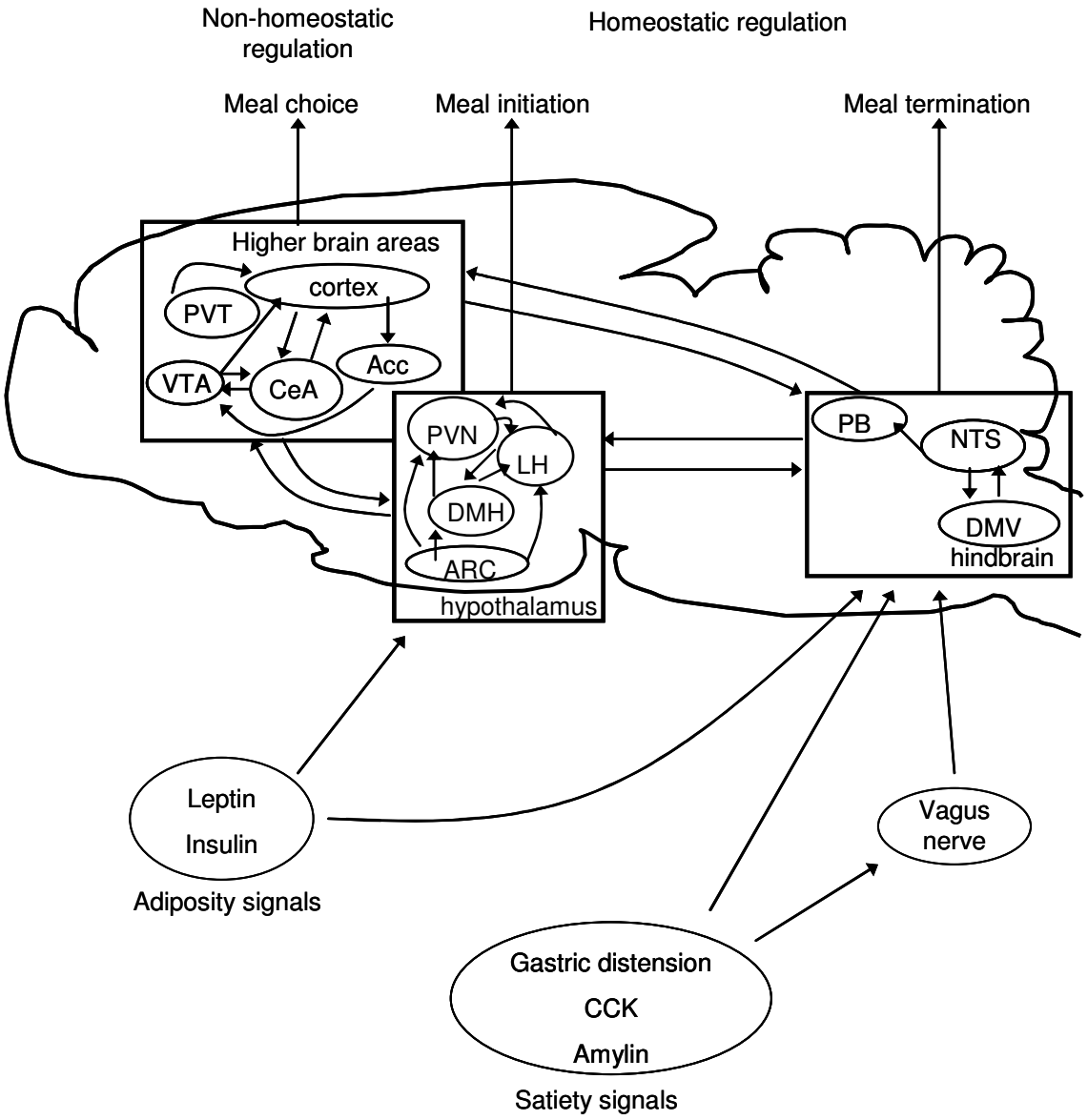

Figure 1: Schematic overview of the neural circuitry involved in the regulation of energy balance. Acc: nucleus accumbens, CeA: central amygdala, PVT: paraventricular nucleus of the thalamus, NTS: nucleus of the solitary tract, DMV: dorsomotor nucleus of the vagus, PB: parabracchial nucleus, VTA: ventral tegmental area. Adapted from (16).

The neurons from the Arc project to areas in the hypothalamus, but also to the brainstem and to higher order systems of the brain (1). The most important hypothalamic downstream targets of the Arc are discussed below.

PVN

The pathway from the Arc to the paraventricular nucleus of the hypothalamus (PVN) is thought to be the most important one for the regulation of energy balance, both for feeding behavior and activation of the sympathetic nervous system. Stimulation of the PVN inhibits food intake, whereas destruction results in hyperphagia and obesity, indicating that the second-order neurons in this area are of an anorexigenic character (17). Consistent with this hypothesis, administration of most of the neuropeptides produced in the PVN reduce food intake. In addition, the second-order neurons of the PVN are stimulated by the POMC/CART neurons of the Arc and inhibited by the NPY/AgRP neurons (18;19).

One of the downstream targets of NPY/AgRP neurons from the Arc are the corticotropin-releasing factor (CRF) producing neurons in the PVN, which are, besides for feeding behavior, important for the regulation of the stress axis and activation of the sympathetic nervous system (20). Also thyrotropin-releasing hormone (TRH) is produced in the PVN, and is implicated in food intake and regulation of the thyroid axis (21). In addition, TRH can increase thermogenesis, sympathetic outflow and metabolic rate (22). Furthermore, oxytocin (OT) (23) and galanin (GAL) (24) are synthesized in the second-order neurons of the PVN.

Thus, the PVN is an important nucleus that is able to modulat both energy intake and energy expenditure by means of several second-order systems.

LH/PFA

The lateral hypothalamus (LH), including the perifornical area (PFA) is also known as the feeding center, since lesioning decreases food intake and electrical stimulation results in hyperphagia (2). Indeed, the neuropeptides in the LH through which energy balance is modulated are orexigenic. One of these neuropeptides is melanin concentrating hormone (MCH), which increases feeding behavior when overexpressed (25). Additionally, MCH mRNA is found to be upregulated after fasting (26). Furthermore, the LH contains orexin expressing neurons, which are, besides in ingestive behavior, involved in arousal (27-29).

MCH and exert their actions via wide projections throughout the brain, of which most are reciprocal. These projections include areas that are involved in salivation and regulation of pancreatic hormones (e.g. preganglionic ganglions in medulla and spinal cord) and areas that are important for arousal and locomotor activity (e.g. locus coeruleus and raphe nucleus) (30). Therefore, the LH may be more involved in aspects leading to feeding behavior than in feeding behavior itself.

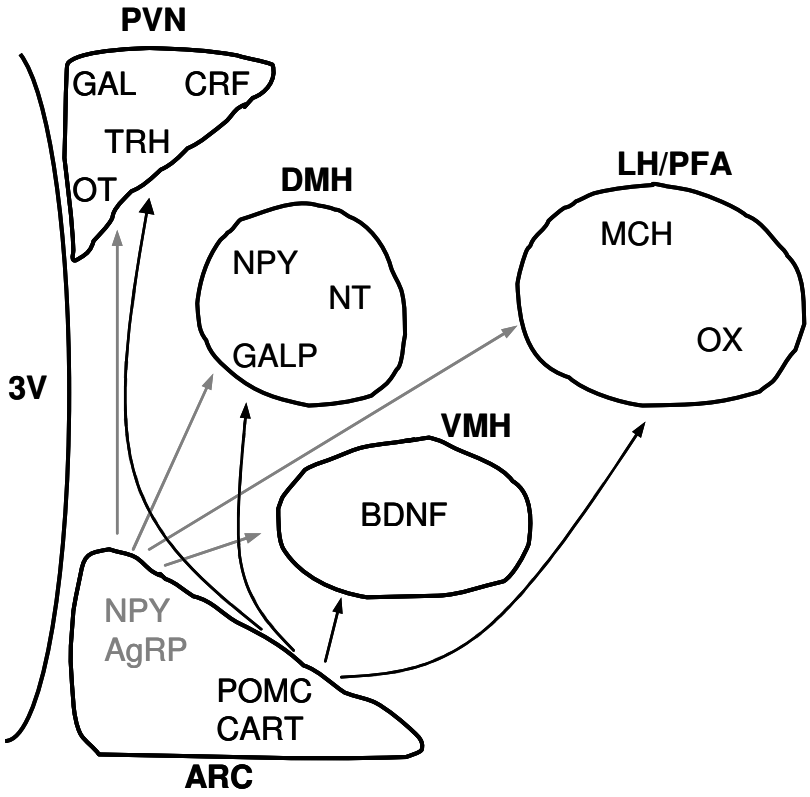

Figure 2: Schematic overview of the most important hypothalamic pathways and neuro-peptides involved in energy homeostasis. 3V: third ventricle, GAL: galanin, GALP: galanin-like protein, OX: orexin, OT: oxytocin, NT: neurotensin.

VMH

The ventromedial nucleus of the hypothalamus (VMH), also known as the satiety center, is also an important hypothalamic region involved in the regulation of food intake. While destruction of this area induces an orexigenic response and obesity, electrical stimulation of the VMH results in the opposite (31;32). The VMH contains neurons that are glucoresponsive and plays an important role in the CRF-mediated regulation of glucose homeostasis (33-35). Selective destruction of the glucoresponsive neurons in the VMH by injection of goldthioglucose causes massive damage and is sufficient to induce hyperphagia and obesity (36;37).

The VMH projects to areas in both the hypothalamus and the brainstem (38). One of the peptides produced by second order neurons in this area that may affect energy balance is brain-derived neurotropic factor (BDNF). BDNF expression is markedly reduced after fasting, and reduction of BDNF signalling or receptor expression increases both food intake and body weight (39;40).

DMH

Finally, also the dorsomedial nucleus of the hypothalamus (DMH) plays a role in homeostatic regulation. Lesion studies of the DMH resulted, similar as for the VMH, in increased ingestive behavior and obesity, although less dramatic (41). The DMH is innervated by both the arcuate nucleus and the brainstem, and sends projections to various hypothalamic areas (e.g. the PVN and LH) (38). In the DMH of the normal rat, low levels of NPY are produced, and this production is increased in both lactating and diet-induced obese rats and in mice that overexpress Agouti or lack the MC4 receptor (42-44). Furthermore, galanin-like peptide (GALP) and neurotensin (NT) expressing neurons in this area may contribute to the regulation of food intake (24;45).

The DMH sends projections to the ventrolateral preoptic area and the LH, which are involved in the regulation of sleep and wakefulness, respectively, but also to the medial preoptic area (involved in the regulation of temperature) and the PVN. Via these projections, the DMH influences circadian rhythms of sleep, feeding, activity, temperature and secretion of corticosteroids (46). Besides the fact that the DMH is critical for circadian rhythms driven by the suprachiasmatic nucleus (SCN), it is also essential for food-entrainable rhythms (47). Thus, the DMH is an important area in the adaptation of several feeding-related behaviours to periods of abnormal food availability.

Hindbrain

While it is hypothesized that the hypothalamus is implicated in the long-term control of energy balance, coordinating meal initiation and frequency, the hindbrain responds to satiety signals from the gut and pancreas (e.g. gastric distension and hormones as cholecystokinin (CCK) and amylin) and thereby controls meal termination and meal size (48).

Like the Arc, the nucleus of the solitary tract (NTS) and adjacent area postrema are located in an area where the blood brain barrier is incomplete (5), and gut peptides from the bloodstream can therefore easily enter the hindbrain. Furthermore, the NTS receives information from the gastrointestinal tract via afferents of the vagus nerve (49;50). The NTS does not only respond to satiety signals, it also receives information about taste (51) and is directly linked to locomotor and oromotor output (52).

NTS neurons have reciprocal connections with the hypothalamus, including the PVN, however, these connections are not essential for meal termination since decerebrated rats maintain the ability to respond normally to satiety signals (53).

The NTS contains neurons that express the leptin receptor (54), and it has been shown that leptin administration to the fourth ventricle inhibits food intake and body weight, similar to third ventricle infusions (55). Similar to the Arc, neurons in the NTS produce NPY or POMC (56;57) which respond to leptin. This indicates that the effects of

leptin on feeding behaviour are not solely mediated by the hypothalamus, but that also the brainstem is involved in long-term control of homeostasis.

Higher order systems

The higher order areas in the brain, including the cortex, nucleus accumbens, hippocampus and amygdala, are important for the non-homeostatic aspects of feeding behaviour (30). Especially the palatability of food is a very important factor in non-homeostatic regulation of food intake. Although palatability is not a key aspect when energy sources are scarce, in a fed state the wanting and liking of a food can overpower the satiety signals, and palatability and variety of food can result in increased ingestive behaviour (58).

Several systems are important for the non-homeostatic regulation of feeding. Opioid agonists, GABA agonists, glutamate antagonists and cannabinoids all increase food intake, preferentially the intake of palatable foods. In addition, in mice that lack the opioids enkaphalin or β-endorphin, the reinforcing property of food is abolished. However, fasting regains this effect (59), again showing the power of the homeostatic systems in periods of energy deficit. Furthermore, also the serotonin system can contribute to the hedonic regulation of food intake (30).

The reward pathway interacts with the homeostatic system to accomplish changes in feeding behaviour. There are, for example, reciprocal connections from the nucleus accumbens with the LH, and also other hypothalamic areas and the brainstem are connected with the higher order systems of the brain (reviewed by (60;61)). Whereas the initiation of feeding is proposed to be controlled by GABAergic outputs from the nucleus accumbens to the lateral hypothalamus, motor activation and motivation to eat (rather than the consumption of food itself) are regulated by dopaminergic inputs in the striatum. In addition, interactions between the opioid system in the striatum, NTS, amygdala and hypothalamus are involved in the palatable evaluation of food (61).

In summary, although the various aspects of feeding behavior (as motivation to eat, food seeking, actual food intake and satiety) are controlled by different areas in the brain (as the hypothalamus, hindbrain and higher order systems), the interactions between these systems are essential for the eventual coordination of food intake.

THE MELANOCORTIN SYSTEM IN ENERGY BALANCE

Melanocortins (MCs) are a family of peptides derived from the precursor protein POMC. Cleavage of POMC leads to the production of adrenocorticotropic hormone (ACTH) and α-, β- and γ-melanocyte stimulating hormone (MSH), but also the opioid peptide β-endorphin (62). In the brain, POMC is expressed in neurons of the Arc and neurons of the NTS (57). These neurons project to a variety of areas in the brain, including the PVN, DMH and LH, the brainstem, amygdala and thalamus (63-68), all of which have been implicated in

energy balance as described before. POMC neurons also project to preganglionic neurons of the spinal cord, suggesting a direct influence on the regulation of energy expenditure via modulation of the sympathetic system (69).

A unique feature of the melanocortin system is that it is also regulated by two endogenous antagonists; agouti (70), an antagonist of the MC1 and MC4 receptor and AgRP (71;72), working as an inverse agonist on the constitutively active MC3 and 4 receptors (73). Agrp is, similar to POMC, expressed in neurons of the Arc, agouti however is not centrally expressed.

Five different melanocortin receptors have been identified in both rodents and humans; the melanocortin 1 receptor (MC1R) - MC5R. The melanocortin receptors differ in function, expression pattern and ligand selectivity (summarized in table 1). The MC3R and MC4R are the predominant receptors in the brain, and these are the melanocortin receptors implicated in the regulation of energy homeostasis. The MC3R is mainly expressed in the hypothalamus (VMH, medial preoptic area (MPO), LH), but at lower levels also in limbic areas (74). Furthermore, the MC3 receptor is expressed in the Arc on POMC neurons. This suggests that the MC3 receptor plays a role as autoreceptor, normally suppressing the activity of POMC neurons (75). The MC4 receptor is expressed in several sites in the brain that have been implicated in energy balance. It is expressed much wider than the MC3 receptor, including in the amygdala, brainstem, cerebellum, cortex, hippocampus, hypothalamus, midbrain and thalamus. In the hypothalamus, expression is found in the PVN, DMH and LH (76-78).

Administration of melanocortins

Central administration

One of the first studies on the melanocortin role in food intake in 1986 revealed that intracerebroventricular (ICV) administration of both ACTH(1-24) and α-MSH inhibit spontaneous feeding (79). Since then, others have found that central injections of α-MSH or synthetic analogues as MTII reduce food intake in both freely feeding and food restricted rats (80-85). However, central MTII also results in conditioned taste aversion (CTA), suggesting that the inhibiting effects on food intake are associated with negative sensations (80;84). The fact that the MC4R selective agonist Ro27-3225 does not elicit CTA, indicates that CTA is mediated by the MC3R, but not via the MC4R (80). While α-MSH and β-MSH both inhibit food intake in fasted and non-fasted rats, γ-MSH does not, (86;87) and β-endorphin even increases feeding when injected ICV (88). Because γ-MSH selectively activates the MC3 receptor, it is suggested that the feeding response of MTII, α-MSH and β-MSH are mainly caused by stimulation of the MC4R. MTII and α-MSH also increase energy expenditure, as shown by enhanced metabolic rate and sympathetic outflow (89).

Table 1: Overview of MCR endogenous ligands, locations and functions, adapted from (16).

MC receptor subtype	*Endogenous ligand (in order of affinity) (110;111)*	*Endogenous antagonist (71;109)*	*Central location (74;78;106-108)*	*Peripheral location (102-105)*	*Central function (97-101)*	*Peripheral function (90-96)*
MC1R	αMSH=βMSH=ACTH >γMSH	Agouti	Periaquaductal grey	Melanocyte, pituitary, placenta, testis, corpus luteum, macrophages, monocytes, neutrophils, endothelium, glioma cells, astrocytes, fibrobasts, keratinocytes, Th cells, NK cells	?	Pigmentation, anti-inflammatory
MC2R	ACTH	Agouti		Adrenals, murine adipocytes, skin, Th cells, NK cells, monocytes, granulocytes		Glucocorticoid production, stress induced lipolysis
MC3R	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
MC4R	αMSH=βMSH=ACTH ≥γMSH	AgRP, agouti	Brainstem, hypothalamus, thalamus, striatum, septum, cortex, hippocampus, limbic system, spinal cord		Body weight regulation, grooming, anti-inflammatory	
MC5R	αMSH>βMSH=ACTH >γMSH	Agouti	Cortex, cerebellum, striatum, midbrain, pons, medulla, olfactory bulb	Pituitary, skin, adrenals, fat cells, smooth and skeletal muscle, bonemarrow, spleen, lymphnodes, thymus, gonadals, uterus, lung, liver, stomach, oesophagus, kidney, mammary glands, exocrine glands, Th cells, NK cells, monocytes, granulocytes	?	Natriuresis, sebumsecretion, preputial lipogenesis

Consistent with the hypophagic effects of MC agonists, central injections of AgRP or synthetic melanocortin antagonists as SHU9119 increase food intake (81;82;112), an effect that can be surprisingly long lasting: a single injection of AgRP can have hyperphagic effects up to seven days (113;114). The acute effects of AgRP on food intake are suggested to be associated with the opioid system, since AgRP-induced hyperphagia can be blocked with the opioid receptor antagonist naloxone (115).

Chronic administration of α-MSH or MTII also results in decreased food intake, although not for the entire infusion period. This is accompanied by weight loss, probably resulting from a decrease in fat mass and improved glucose uptake recovery (83;116;117). Likewise, chronic infusion of AgRP or synthetic antagonists results in a continuous increase in food intake and an accelerated weight gain, together with increased brown (BAT) and white adipose tissue (WAT) mass and plasma leptin and insulin levels (71;116;118-120). Simultaneously, a reduction in energy expenditure is observed after chronic infusion of AgRP, as shown by a decrease in oxygen consumption and a suppression of uncoupling protein (UCP) I in brown adipose tissue (119-121). Although body weight is not altered in pair-fed animals, adipose tissue mass and plasma concentration of leptin and insulin are increased, and UCPI is still suppressed. This indicates that these effects are independent from food intake (119;121). Finally, a reduction in AgRP by RNA interference results in increased metabolic rate and reduced weight gain (122).

Site-specific administration

Studies investigating the site of action of (an)orexigenic effects of MC receptor ligands within the hypothalamus and amygdala identified the PVN, DMH and MPO as primary sites (123).

Local injection of the MC4 receptor agonists MTII or αMSH in the PVN results in a reduction of food intake in mice as well as in rats. Interestingly, the effects on food intake are only observed when feeding is stimulated, for instance by dark onset, fasting, or NPY injection (82;124;125). Similarly, MC4R antagonists as AgRP, SHU9119 and HS014 stimulate feeding when administered directly into the PVN (82;124;126;127). Again, these antagonists are merely effective when meal initiation is triggered, suggesting that signaling via the MC4R in the PVN is implicated in meal duration rather than meal initiation.

As mentioned above, a second hypothalamic site that shows involvement of the melanocortin system in appetite is the DMH. NDP-MSH and AgRP administration to the DMH result in hypophagia or hyperphagia, respectively (123;128). In the same way as for the PVN, the effects of the ligands are only observed from 4 hours after injection, unless feeding is stimulated by fasting (128). Furthermore, AgRP administration in the DMH increases sucrose intake, while it does not affect the intake of an isocaloric product as corn

starch, indicating that the actions of the MC system are influenced by the palatability of a diet (128).

Grill *et al.* found that 4th ventricle administration of the MC4R ligands MTII and SHU9119 has comparable effects on food intake as administration to the lateral ventricle. This suggests that the caudal brainstem contributes to the effects of the MC system on appetite (129). More specifically, Williams *et al.* injected MTII directly into the dorsal vagal complex (DVC), which had a reducing effect on food intake and body weight, while SHU9119 stimulated food intake, demonstrating that the brainstem MC effects on appetite are mediated by the DVC (130).

Furthermore, while effects on food intake are absent after administration of the MC4 agonist NDP-MSH to the central amygdala (CeA), the antagonists AgRP or HS014 do increase food intake, although to a lesser extent than injection in the PVN, DMH or MPO (123;126). MC4 agonists or antagonists injected in the LH, Arc, VMH and nucleus accumbens have little or no effect on food intake (123;126).

Transgenic models

POMC

Transgenic overexpression of POMC resulting in a six-fold overexpression of POMC mRNA has no effect on food intake and weight of fat pads. It does, however, result in a slight decrease in body weight gain and also in reduced fasting-induced hyperphagia. POMC overexpression partially corrects the obese phenotype of leptin deficient mice, as shown by a normalized glucose metabolism and a phenotype intermediate between $Lep^{ob/ob}$ and $Lep^{+/+}$ mice (131).

Comparable results are obtained after transgenic overexpression of the N-terminal part of POMC. Because N-terminal POMC needs further processing to produce active peptides, it is assumed that this strategy will only result in an overexpression of active α- and γ_3-MSH in cells that normally are able to produce these peptides. Overexpression of the N-terminal part of POMC results in a decrease in body weight gain of male mice, again without an effect on food intake. Nevertheless, fasting-induced refeeding is not altered in these mice. Although plasma glucose levels were not altered in mice with an overexpression of α- and γ_3-MSH, these mice do show decreased fed and fasted insulin levels and have improved insulin sensitivity (132).

Li *et al.* were able to reduce food intake and body weight of obese, aged rats by injecting a recombinant adeno-associated virus (rAAV) encoding POMC in the basolateral hypothalamus. Although the hypophagia was transient (during only 19 days), the loss of body weight remained stable until the end of the study (42 days), even when rats had regained their normal food intake. The decrease in body weight was accompanied by a reduction in body fat and plasma leptin levels, and improved glucose metabolism.

Furthermore, rats with a viral induced overexpression of POMC had increased UCPI levels (133). Together with the fact that POMC expression is reduced in aged rats (134;135), this suggests that reduced activation of the melanocortin system is involved with age induced obesity, which is besides with increased visceral fat, also characterized by decreased levels of UCPI and impaired glucose metabolism (136).

Agouti

Dominant mutations in the *agouti* gene result in the yellow agouti obese mouse syndrome, characterized by yellow pigmentation, adult-onset obesity, hyperphagia, hyperinsulinemia and increased lean body mass (137). Two of these mutations, the lethal yellow (A^y) (138) and the viable yellow (A^{vy}) (139) resulting in a continuous expression of agouti in all tissues, including the brain, have been widely studied (137;140;141). Since agouti has no affinity for the MC3 receptor, the effects through which ectopic agouti expression in the brain results in obesity are linked to the MC4 receptor (81;100;142-144).

Yellow agouti mice display an increased feed efficiency as shown by the increased weight gain per gram food consumed, however this is abolished by food restriction (145). Although an increased feed efficiency suggests that, besides an increase in food intake, also a decrease in energy expenditure contributes to the obese phenotype of yellow agouti mice, this remains to be investigated.

Obese A^y mice have an enhanced preference for fat intake and also gain more weight when fed a high fat diet when compared to wildtype littermates (146). Additionally, when fed a high sucrose or high fat diet for 3-5 weeks, yellow, but not lean agouti mice show a decreased glucose tolerance and an impaired insulin sensitivity of the adipocytes (145).

Finally, A^{vy} have an increased number of pancreatic β-cells, which can, via the excess of plasma insulin levels, account for the increased lipid deposition and weight gain of these animals (147).

AgRP

Overexpression of AgRP results in a phenotype that is in many features similar as that observed in the obese yellow mice. Animals show increased food intake and rapid onset obesity (71;148). Furthermore, as A^y and A^{vy} mice, AgRP transgenic mice display increased lipid storage in adipose tissue and liver, hyperplasia and hypertrophy of the pancreatic islets, and increased levels of plasma glucose and insulin (148).

Knock out models

$POMC^{-/-}$

POMC deficient mice show increased food intake, body weight and plasma levels of leptin, which can be explained by the lack of endogenous melanocortins (149-151).

Hyperphagia and obesity are even more pronounced when animals are fed a high fat diet, indicating that POMC$^{-/-}$ mice are not able to adjust their metabolism to high caloric feeding (151). Interestingly, the obese phenotype in POMC-mutant mice seems to result mainly from altered lipid metabolism since the weight gain, particularly in fat tissues, is much greater than the increase in feeding behavior (151). Furthermore, also reduced energy expenditure as indicated by reduced basal oxygen consumption can contribute to the obese phenotype of the POMC$^{-/-}$ mice (149).

AgRP$^{-/-}$

Surprisingly, young AgRP$^{-/-}$ mice do not have an obvious phenotype, suggesting that AgRP does not play an essential role in the regulation of energy homeostasis. Under ad libitum feeding conditions, they show normal food intake, normal body weight and normal endocrine parameters (152). However, after a 24h fast, animals lacking AgRP do have an impaired hyperphagic response, indicating that AgRP is important under conditions when energy is scarce (153). Nevertheless, when AgRP$^{-/-}$ mice age, they show decreased body weight and adiposity, due to increased locomotor activity, body temperature and metabolic rate. Food intake however remains normal (154).

Recent data indicate that interpretation of results obtained from knockouts is complicated by developmental compensations. In transgenic lines where the human diphteria toxin receptor was expressed in the AgRP/NPY neurons of the arcuate nucleus, diphteria toxin was injected in various time points to selectively ablate the AgRP/NPY neurons at different stages of life. Although removal of the AgRP/NPY neurons in neonates did not affect feeding behavior and body weight gain, administration of diphteria toxin in adult mice caused a dramatic reduction of food intake and a lean phenotype (155;156).

MC3R$^{-/-}$

Mice lacking the MC3R are slightly hypophagic (97), which can be explained by increased melanocortin signalling due to the loss of MC3R as autoreceptor on POMC neurons in the Arc. Despite the normal, or even decreased food intake, MC3R have an increased adipose mass. However, due to a reduced lean mass no differences in body weight are observed (97;157). MC3R$^{-/-}$ mice also show an impaired glucose homeostasis (158).

MC3R$^{-/-}$ mice respond normally to MTII administration (97;159), and peripheral injected CCK inhibits food intake as it does in wild type animals (160). They also have normal plasma ghrelin levels, however the feeding response to peripheral injected ghrelin is blunted (161).

Although overall energy expenditure is not significantly changed in animals missing the MC3R (97;157), they do show a reduction in running wheel or spontaneous activity in

the dark phase (97;157;162). This seems contradictory, nevertheless, it is possible that the differences in metabolic rate are too small to be measured.

When MC3R$^{-/-}$ mice are weaned on a moderate or high fat diet, females display a slightly increased weight gain, however, food intake is normal, suggesting a difference in metabolism (97;157). On a purified high fat diet MC3R$^{-/-}$ mice modestly increase their food intake without a change in body weight. This change in food intake is limited to the light phase, which suggests that the MC3 receptor plays a role in circadian rhythm of food intake (162). Respiratory exchange ratio (RER) is moderately increased in both normal and high fat diet fed MC3R$^{-/-}$ mice, which might indicate a reduction of fatty acid oxidation (157;162).

Due to the increase in fat mass, together with a lack of distinct effects on food intake caused by ablation of the MC3R, it can be suggested that the MC3R is more important in energy expenditure than for the regulation of feeding.

MC4R$^{-/-}$

Disruption of the MC4 receptor results in a phenotype similar as the yellow mouse syndrome observed in the (A^{y}) and (A^{vy}) mice. MC4R$^{-/-}$ mice display maturity onset obesity characterized by hyperphagia, increased adiposity, normal lean body mass, hyperinsulinemia and hyperleptinemia. Although female MC4R$^{-/-}$ mice have normal plasma glucose levels, male knockouts are hyperglycemic from 10-14 weeks of age. Furthermore, absence of the MC4 receptor results in increased longitudinal growth. MC4R$^{+/-}$ mice show an intermediate phenotype with respect to body weight and food intake, suggesting a gene dosage effect (100;163).

Peripheral as well as central leptin does not reduce food intake in MC4R$^{-/-}$ mice as it does in wild type animals, indicating that MC4R$^{-/-}$ mice are leptin resistant. The fact that young, non obese MC4R$^{-/-}$ mice do respond to leptin, although with a blunted response (159), demonstrates that besides the MC4 receptor, other systems act downstream of leptin in leptin's effect on food intake. However, the fact that leptin administration in non obese female MC4R$^{-/-}$ mice is not able to increase UCPI mRNA as it does in wild type mice (164), suggests that for some of the effects of leptin, intact MC4 receptor signaling is essential.

While MTII administration in wild type controls inhibits fasting-induced refeeding, this is not true for MC4R$^{-/-}$ mice (159). Furthermore, MTII also does not inhibit nocturnal feeding in freely feeding MC4R$^{-/-}$ mice (159) or 24h feeding (163) as it does in wild types. This indicates that the central melanocortin peptides that inhibit appetite act through the MC4 receptor. Centrally administered AgRP however does seem to induce hyperphagia in MC4R$^{-/-}$ mice. 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 for instance the MC3 receptor. (165;166). The MC4 receptor seems not to be involved in the

orexigenic or anorexigenic effects of NPY, peptide YY (PYY), orexins and urocortin, as the response to these neuropeptides on food intake is normal in MC4R$^{-/-}$ mice (159;167).

MC4 receptors involved in appetite are not only located in the hypothalamus, but also in the brainstem, as demonstrated by Fan *et al*. They showed that peripheral CCK was not able to reduce fasting induced food intake in MC4$^{-/-}$ mice. Administration of SHU9119 in the third or fourth ventricle revealed that the CCK-mediated inhibition of food intake is controlled by MC4 receptors in the brainstem and not the hypothalamus (160).

Since MC4$^{-/-}$ mice get obese before they become hyperphagic, it is suggested that obesity in mice lacking the MC4 receptor is not only due to increased food intake, but also to hypometabolism. Although body temperature of MC4$^{-/-}$ mice is normal, locomotor activity of male MC4$^{-/-}$ mice is reduced in the dark phase (164), indeed indicating a reduced energy expenditure. Studies on oxygen consumption levels in obese animal models are difficult to interprete, since metabolic activity in fat tissue is lower than in other tissues as for instance muscle (168). Nevertheless, MTII is not able to increase metabolic rate in MC4$^{-/-}$ mice, suggesting that the MC4 receptor indeed is necessary for the regulation of metabolism (163). Additionally, when MC4$^{-/-}$ mice are pair-fed to wild type littermates, their body weight, fat mass and leptin levels are intermediate of that of wild types and ad libitum fed MC4$^{-/-}$ mice (164), which also implies that hyperphagia is not the only reason for the increased body weight of MC4$^{-/-}$ mice.

When given the choice between a high fat, high protein and high carbohydrate diet, wild type animals reduce specifically the intake of the high fat diet, while the intake of high protein and high carbohydrate derived calories remains unchanged. This effect is absent in MC4$^{-/-}$ mice, suggesting that the MC4 receptor is necessary for the MTII induced reduction of fat, but not protein or carbohydrate intake (169).

Despite the fact that food deprivation has comparable effects on body weight loss in MC4$^{-/-}$ and wild type mice, increased dietary fat has different effects in mice lacking the MC4 receptor compared to controls. When exposed to a high fat diet, MC4$^{-/-}$ mice display an increased caloric intake, which is, unlike in control animals, not normalised after 48 hours. Together with an enlarged feed efficiency, this results in an even more increased body weight gain. Additionally, while normal animals increase their oxygen consumption on a high fat diet, this effect is absent in MC4$^{-/-}$ mice. This indicates that the MC4 receptor is required for a normal metabolic and behavioral response to increased dietary fat (170).

THE NPY SYSTEM IN ENERGY BALANCE

NPY and PYY are members of the pancreatic polypeptide family. PYY is produced peripherally, predominantly in L-cells of the distal gastrointestinal tract (171;172). It is involved in gut motility, gastric emptying and secretion of gastric and pancreatic enzymes (173). PYY is released following food intake (171) and although central administration of

PYY increases food intake, peripheral injections inhibit food intake and body weight gain (174-176).

NPY is widely expressed in the brain and implicated in a variety of physiological processes, including anxiety, reproduction and energy homeostasis. High numbers of NPY expressing neurons are found in the arcuate nucleus and the brainstem, although also in the DMH NPY can be produced (56). In the Arc, NPY is co-localized with AgRP (7) in neurons that project to numerous hypothalamic regions that play a role in energy balance, as the PVN, PFA, LH and DMH, as well as to the brainstem (56;177-179). NPY neurons from the brainstem also project to various hypothalamic areas (among others the MPO, PVN, DMH), strengthening the vision of a tight regulation between different brain areas in energy balance (179;180).

To date, 5 different NPY receptors (YR) have been cloned, all belonging to the GPCR family and capable of coupling to G_i, thereby inhibiting adenylate cyclase. PYY and NPY have a similar affinity for all Y-receptors. The Y1, -2, -4 and -5 receptors are found to be active in both humans and rodents, the Y6 receptor, however is only functional in the mouse and rabbit and even absent in the rat (181). Due to the lack of highly selective agonists or antagonists, it is difficult to distinguish the contribution of the different receptor subtypes to the regulation of energy balance.

The Y1 receptor subtype is a postsynaptic receptor, found in almost all hypothalamic subregions in the rat, with highest expression levels in the Arc and SON (182). In the Arc, Y1 receptors are predominantly expressed on POMC neurons, suggesting an inhibitory role in the expression of POMC (183). Y2 immunoreactivity in the hypothalamus is found among others in the arcuate nucleus, DMH, LH and MPO Only few Y2 positive cells are found in the PVN (184;185). In the arcuate nucleus, Y2R is almost exclusively found on the soma of NPY containing neurons, indicating an inhibitory role as autoreceptor for this subtype (183). Peripheral administered PYY is thought to act via the Y2 receptors in the Arc, while central PYY acts via the Y1 and Y5 receptors further in the hypothalamus, thereby explaining the opposing effects observed after injection of PYY. Most hypothalamic areas express only restricted amounts of the Y4 subtype, high Y4 expression is limited to the PVN (186). Distribution of the Y5 receptor in the hypothalamus is similar to the distribution of the Y1 receptor, with high levels of Y5 mRNA found in almost all hypothalamic areas (182;187-189).

Although all the receptor subtypes have been found in hypothalamic areas important for energy homeostasis, signalling via the Y1 and Y5 receptor seems to be most likely responsible for the feeding effects of NPY (187;190-192), while the Y2 receptor serves as a regulatory feed back loop.

Administration of NPY

Central administration

ICV administration of NPY increases food intake (193;194), by increasing meal frequency and meal duration. However, it decreases eating speed, thereby not altering meal size (195). In line with this it is suggested that NPY affects the appetitive, but not the consumatory phase of ingestive behavior (196;197). Furthermore, acute NPY injections increase insulin secretion, an effect that is partly independent from food intake (198-203).

In addition, energy expenditure is also affected by acute NPY administration. ICV NPY administration reduces body temperature (204;205). This is consistent with a decrease in thermogenesis in brown adipose tissue, mediated by a suppression of sympathetic activity to interscapular brown adipose tissue and a reduction of UCPI mRNA (206-208). Besides, activity in the home cage or the open field is suppressed (209;210). Since ICV administration of a Y5 selective agonist is reported to reduce oxygen consumption (211), this receptor subtype is thought to be involved in the NPY-mediated control of energy expenditure.

When ICV infusion of NPY is continued for a week, the combination of a persistent increase in food intake and reduction in energy expenditure results in obesity, characterized by elevated plasma concentrations of corticosterone, leptin and insulin, and increased lipogenesis in liver and adipose tissue (121;203;212-214).

Most of the obesogenic effects observed after chronic administration of NPY are not dependent on food intake, since pair-fed animals infused with NPY also show increased adiposity, plasma corticosterone, leptin and insulin levels and insulin resistance, although body weight itself remains normal. This increased energetic efficiency can be explained by a reduction in UCPI activity in BAT (121;203).

Consistent with the increase in food intake observed after central administration of NPY, infusion of antisense oligonucleotides induces hypophagia and slower weight gain. The decrease in food intake is however due to a decrease in meal size and duration, while eating speed is unaltered (215).

Site-specific administration

Feeding responses to NPY are found after injection into the PFA, PVN, LH and VMH (216;217). Therefore, these sites are assumed to be the prior areas involved in NPY's actions in the central regulation of energy homeostasis. However, NPY signalling in other sites also increases food intake and may also affect other aspects of the energy balance.

NPY is synthesized in neurons of the arcuate nucleus. Suppression of NPY by injection of antisense oligonucleotides in this area results as expected in a decrease in food intake. In a free choice paradigm, decreased levels of NPY specifically reduce the intake of fat and carbohydrates, while protein intake is not affected (218). Chronic suppression of

NPY induced by AAV mediated expression of antisense NPY cRNA for 50 days results in a continuous decrease in food intake and thereby a reduction in weight gain. In this set up also fasting induced food intake is inhibited (219).

Most local NPY studies have focused on the PVN, since the PVN contains extremely high concentrations of presynaptic NPY (178). A single injection of NPY into the PVN increases food intake, preferentially from a carbohydrate source (216;220;221). Also energy expenditure is affected, as shown by decreased thermogenesis of BAT (207;208) and a reduction in BAT UCPI expression (222). Effects on body temperature however are contradictory, with both hyper- and hypothermia reported (204;205;223). Furthermore, NPY signalling in the PVN is suggested to cause a shift towards carbohydrate utilization and fat synthesis, since respiratory quotient is increased after administration of NPY to the PVN (224). Repeated daily injections of NPY in the PVN of female rats on a high fat diet result in sustained hyperphagia and consequently obesity (225). Consistent with the antisense nucleotide injections in the Arc, in a choice diet both carbohydrate and fat intake are increased, while protein intake is unaltered (226). It remains to be determined however what the effects are of long-term NPY administration in the PVN on energy expenditure.

By mapping the hypothalamic areas involved in the feeding response of NPY, Stanley *et al.* found that not the PVN, but the PFA elicits the strongest feeding response of NPY (217). NPY injections directly in the PFA result in similar feeding patterns as fasting does, increasing both meal size and frequency (227). Nevertheless, despite the huge effects of NPY on energy intake in this area, the PFA seems not to be involved in the regulation of energy expenditure, since body temperature, locomotor activity and respiratory quotient are not altered after NPY injection in this area (204;205;223;224).

Although injections of NPY in the LH, VMH and MPO all result in an increase in food intake, effects on energy expenditure vary. Sympathetic nerve activity to BAT is not altered after NPY injection to the LH, but increased after injection to the MPO, while body temperature is reduced after administration to either area (204;205;208). In the VMH, thermogenesis is not influenced by NPY administration, as shown by a normal body temperature, and normal sympathetic activity to BAT (204;205;208). This emphasizes the fact that one neuropeptide can elicit variable actions in different hypothalamic areas.

The amygdala is another region rich in NPY receptors, and involved in the anxiolytic actions of NPY (228). NPY administration directly into the amygdala however does not alter food intake in either fed or fasted rats (228;229). Even when animals are given the choice between a high and low fat diet, their total caloric intake remains comparable to that of saline injected animals, however, NPY injection in the amygdala does reduce their preference for the high fat diet (229). Together, this suggests that the amygdala does not play a role in NPY-mediated regulation of energy balance, but may be involved in food choice.

Transgenic models

NPY

Global overexpression of NPY in transgenic mouse and rat models does not affect food intake or body weight when animals are fed on regular chow (230-233). Only when bred to homozygosity and fed a high sucrose diet, mice overexpressing NPY in the central nervous system (amongst others in the arcuate nucleus) displayed hyperphagia and increased body weight gain and energetic efficiency. In addition, these mice had increased levels of plasma glucose and insulin, but a normal glucose tolerance (234). It is possible that the lack of effects of NPY overexpression is due to a developmental change, compensation by counter-regulatory mechanisms or only limited overexpression. As mentioned earlier, NPY/AgRP neurons can be destroyed without changing feeding behavior and body weight in neonatal mice, but not in an adult stage (155;156), indicating that indeed the systems involved in energy balance show more plasticity during development then later in life.

Knock out models

$NPY^{-/-}$

NPY deficient mice show, similar to $AgRP^{-/-}$ mice, no obvious phenotype. $NPY^{-/-}$ mice have a normal food intake, body weight and amount of adipose tissue. Furthermore, also their endocrine profile looks normal, with no changes in plasma levels of leptin, insulin, glucose or corticosterone (235;236). Besides, according to their normal body weight, mice lacking NPY have normal energy expenditure, as shown by a similar basal oxygen consumption, body temperature and physical activity as wild type littermates (237). However, when more carefully examined, NPY deficient mice do have an attenuated feeding response to both leptin administration and fasting (235;238;239). $NPY^{-/-}$ mice also show an increased latency to eat at dark onset, also after fasting. This results in a decrease in the first 4h food intake in the dark period (240). Together with the blunted feeding response to a fast or leptin administration, this indicates that NPY is essential for a normal response to feeding cues, and thus implicated in the initiation of feeding. Furthermore, disruption of NPY results in an increased feeding response to central AgRP administration, without showing a change in MTII sensitivity. Simultaneously, normal prefasting AgRP levels and normal AgRP upregulation after fasting is observed, suggesting that compensations by AgRP are not responsible for the relatively normal phenotype of the $NPY^{-/-}$ mice (166). This vision is strengthened by the fact that also AgRP and NPY double knockouts have a normal food intake and body weight (152). Apparently there are other compensatory pathways activated when the NPY gene is lost.

All the original $NPY^{-/-}$ mice studies are performed on a 129-C57Bl/6J mixed background. The 129 mouse strain is resistant to obesity, whereas the C57Bl/6J strain is more susceptible to obesity (241;242). When examined on a C57BL/6J background, $NPY^{-/-}$ mice also

show a normal food intake. However, a normal to a mild increase in body weight and adipose tissue mass and a reduction of lean body mass is observed. Also on this background, NPY deficiency does not affect basal energy expenditure, and does reduce fasting induced hyperphagia, especially in the light phase (243;244). Nevertheless, in this strain AgRP is increased in the fed state of $NPY^{-/-}$ mice and does not increase further after fasting. In addition, on a C57BL/6J background, $NPY^{-/-}$ mice are resistant to obesity. This is due to a decrease in high fat intake, especially in the dark phase (243).

Effects of deletion of NPY or the receptor subtypes on energy balance are summarized in table 2.

$Y1^{-/-}$

Because the Y1 receptor is thought to be one of the main NPY receptors mediating the effects of NPY in energy balance, it was expected that deletion of Y1 would result in a lean phenotype and a decrease in food intake. Nevertheless, $Y1^{-/-}$ mice (especially females) display a phenotype that is much alike the metabolic syndrome, demonstrated by obesity, insulin resistance and a susceptibility to develop hypertension (245-247). The insulin resistance results in a dramatic increase of weight gain, adiposity and plasma levels of leptin and glucose compared to wildtypes when fed a high fat diet (247).

Because food intake is normal, or even slightly reduced, an increase in energy intake is not the cause of the obese phenotype of the $Y1^{-/-}$ mice (245;246). Therefore, it is logical to assume that the increased adiposity results from a decrease in energy expenditure. Indeed, although $Y1^{-/-}$ mice display a normal body temperature and oxygen consumption, they are less active and have reduced movement associated thermogenesis. Furthermore, also metabolic rate is reduced, and UCP2 expression in white adipose tissue is reduced, suggesting an increased energetic efficiency (245;246).

The fact that NPY induced feeding is markedly reduced in animals lacking the Y1 receptor confirms the importance of this receptor subtype in the feeding effects of NPY (248).

$Y2^{-/-}$

Initial studies on deletion of the Y2 receptor reported mice with an increased body weight and food intake. With regard to energy expenditure, $Y2^{-/-}$ mice have normal thermogenesis, but increased light and dark phase locomotor activity. This is in agreement with the role of the Y2 receptor in an inhibitory feedback loop. These mice show a normal feeding response to both fasting and NPY administration, but insensitivity to leptin (185). The increased adipose tissue mass and basal plasma leptin levels of the $Y2^{-/-}$ mice might explain the decreased response to leptin, however it is also possible that the Y2 receptor is involved in leptin sensitivity.

In contrast to the $Y2^{-/-}$ mice described above, germ line $Y2^{-/-}$ mice generated by Sainsbury *et al.* show a decreased body weight gain. Although females do increase their food intake, males have a decreased food intake at younger age and a normal intake when they are adult. Both sexes have a normal endocrine profile, but a pattern of neuropeptide expression in the arcuate nucleus that is typical for a negative state of energy balance, with increased levels of AgRP and NPY, and decreased levels of POMC and CART (249).

The same group also generated a conditional Y2 deficient line. Hypothalamic specific deletion of Y2 using a Cre expressing adenovirus in this line results in a decrease in body weight gain and an increase in food intake. Similar as the germ line knock outs, adult suppression of Y2 receptors in the hypothalamus upregulates NPY and AgRP in the arcuate nucleus, however, in these mice also POMC mRNA is increased (249).

$Y4^{-/-}$

Although the Y4 receptor is not thought to play a major role in the regulation of energy homeostasis, deletion of the Y4 receptor results in a lean phenotype and decreased food intake (250). It is however possible that not the removal of Y4 itself, but compensations in the rest of the NPY system are responsible for the reduced body weight of $Y4^{-/-}$ mice animals.

Double knock outs missing both Y2 and Y4 show a strong increase in feeding behavior, but do retain the lean phenotype of $Y4^{-/-}$ mice, with reduced body weight and adiposity and normal plasma levels of leptin and insulin (251).

$Y5^{-/-}$

Disruption of the Y5 receptor results in late onset obesity, characterized by increased body weight and food intake and elevated fat storage in the liver and adipose tissue. The phenotype is more distinct in males than in females. Animals have a normal body temperature, suggesting that metabolic rate is unchanged (191).

Although the feeding response normally observed after a high dose of NPY is reported to be reduced, others have found that either an acute injection or chronic infusion of NPY increases food intake in $Y5^{-/-}$ mice similar as it does in wild type littermates (191;248;252). Together with the fact that NPY administration reduces POMC and NPY mRNA in the Arc in both wild types and $Y5^{-/-}$ mice, this indicates that the Y5 receptor is not the only receptor mediating the effects of NPY in feeding behavior.

$Y1^{-/-}2^{-/-}4^{-/-}$

Triple deletion of the Y1, Y2 and Y4 receptor prevents the hyperinsulinemia that is observed after viral-mediated overexpression of NPY in the hypothalamus of both wildtype and $Y1^{-/-}$, $Y2^{-/-}$ and $Y2^{-/-}4^{-/-}$ double knockouts, but does not prevent the NPY-induced

hyperphagia and obesity, suggesting that all these Y receptor subtypes are involved in the regulation of insulin secretion. The fact that these animals are still hyperphagic and obese, despite the deletion of three Y receptors, provides more evidence for the implication of the Y5 receptor in the regulation of food intake. This study also suggests that Y receptors indeed can compensate one another after deletion of one subtype (253).

Table 2: Summary of phenotype of NPY or Y-receptor deficient mice

-/-	BW	FI	Npy induced feeding	Leptin induced feeding	Fast induced feeding	Energy exp
Npy	=/↑	=	=	↓	↓	=
Y1	↑	=/↓	=/↓		↓	↓
Y2	↑/↓	↑	=	↑	=	↑
Y4	↓	↓				
Y5	↑(aged)	↑(aged)	=/↓			=

RAAV: A VIRAL BASED APPROACH FOR GENE DELIVERY TO THE BRAIN

Although a lot is known by now about the role of melanocortins and NPY in the regulation of energy homeostasis, there are some discrepancies between the results of transgenic and knockout studies on one side and central administration of the ligands of these systems on the other side. Whereas administration of AgRP or NPY into the third ventricle has striking effects on food intake and, when infused for a longer period also on body weight, both $NPY^{-/-}$ and $AgRP^{-/-}$ mice show relatively normal feeding behavior.

One aspect that is often ignored however, is that ICV administration of a ligand activates simultaneously all its receptors at various locations in the brain, which is not a normal physiological response. A similar situation occurs in transgenic or knockout models, where a gene is either overexpressed in many regions at once, sometimes even in areas where it is normally not expressed, or deleted in all places. This may lead to secondary effects, which may not represent the normal function of a gene. Leptin for example plays a very important role in the perinatal development of hypothalamic circuitries involved in energy balance. In leptin-deficient mice the pathways projecting from the Arc to its target nuclei are severely reduced (254;255). The obese phenotype of leptin deficient mice may therefore not only be due to the absence of leptin, but also to the reduced amount of AgRP/NPY and POMC neurons.

Another issue one has to keep in mind is that the mammalian brain exhibits a high plasticity, especially during development. It is possible that changes in gene expression (for instance by transgenic overexpression) at some stage in development result in the activation of counter-regulatory systems, and therefore changes in behavior are masked by the compensational actions of other genes.

Furthermore, most pharmacological studies are performed with icv administration of agonists or antagonists, elucidating only overall effects of a neuropeptidergic system, and only few have focused on region specific functions of NPY or MCs. This is partly due to the fact that local long-term stimulation or suppression is hard to achieve. The simplest method would be to infuse ligands into a specific area via a permanent cannula in the brain; however it is not feasible to reliably infuse ligands locally in the brain for more than a week.

In this thesis we used a gene therapy-based approach as an alternative strategy to study the specific role of the MC and NPY systems in different hypothalamic nuclei on the regulation of energy balance.

AAV in gene therapy

Gene therapy is a relatively new strategy in current research. Besides offering new treatment possibilities for many genetic and non-genetic diseases, it can be used as a tool for basic neurobiological research. It is used to achieve stable gene expression in a tissue of choice for as long as required. Both overexpression of a specific gene, and suppression of the gene by RNA interference, at any time from development on or in adult stages is possible. It would therefore be a good strategy to use for further studies on the local functions of neuropeptides in energy balance.

The fact that the adeno-associated virus (AAV) is derived from a non-pathogenic family, and that AAV-mediated transduction elicits minimal or no toxicity and induces long-term, stable gene expression in the absence of an obvious immune response, makes it an excellent candidate for gene therapy among a variety of viral and non-viral delivery systems (256;257). Moreover, AAV is one of the few systems that can efficiently transduce a wide spectrum of both dividing and non-dividing cells (258-264) and is therefore often used as a tool for gene delivery to the brain.

rAAV vectors

AAV is a single stranded, non-enveloped DNA virus that belongs to the family of *Parvoviridae*. Wild type AAV integrates preferentially in the long arm of human chromosome 19 (265-267), however, this feature is lost in recombinant AAV. An advantage of AAV as a gene delivery vehicle is that AAV is dependent on co-infection by a helper virus such as adeno- or herpes virus for efficient replication (268;269), making it relatively safe to work with.

The genome of AAV consists of two open reading frames, containing the REP (involved in replication and regulation of gene expression) and CAP (encoding the three viral coat proteins) genes, flanked by two inverted terminal repeats (ITR), which are necessary for packaging, replication and integration (reviewed by (270)). In rAAV vectors, all viral genes but the ITRs are removed and replaced by the transgene.

The genome is highly conserved among the approximately 35 serotypes, from which almost ten have been engineered into recombinant vectors (271-276). The most common serotype used is AAV2. The primary receptor for AAV2 is the cell surface heparin sulfate proteoglycan (HSPG), which acts in synergy with co receptors in the attachment of the virus to and entry into a cell (277-279). HSPG is expressed on a wide variety of cells, explaining the broad spectrum of mammalian cells that can be infected by AAV2 (280-282). Differences in tropism of the various serotypes are probably due to differences in receptor binding and entry features.

Although 35-80% of the human population maintains antibodies to AAV2 (283-285), which may limit therapeutic effects of rAAV-based therapies, only a minimal immune response is observed when AAV2 is injected in naïve rodents (286-289).

rAAV mediated gene transfer to the central nervous system

The use of rAAV as gene delivery tool in the central nervous system has been extensively reviewed elsewhere and will therefore only be discussed shortly (290-292).

When injected into the brain, AAV2 transduces predominantly neurons, however, occasional infection of microglia and oligodendrocytes is also observed (261;280;293-296). Transduction efficiency depends markedly on the injected area, with high efficiencies reached in the hippocampus, but much smaller numbers of transducable cells for example in the striatum (280). Furthermore, transduction patterns vary with the serotype used. AAV1, AAV5 and AAV8 all result in high transduction efficiency and a wide distribution of transduced cells and with both AAV5 and AAV8 transgene expression at distance from the injection site is observed, indicating possible tracing capacities (295;297). While AAV1 similar as AAV2 preferentially infects neurons, AAV5 also transduces a significant amount of astrocytes and ependymal cells, and AAV4 almost exclusively infects ependymal cells (295;296).

With the use of AAV, long-term stable transgene expression up to 25 months can be achieved in the brain; however, duration of expression varies with the promoter used and the area infected (280;286;298;299). The use of the neuronspecific promoter "neuron specific enolase" (NSE) seems to result in a long-term expression of the transgene, while cytomegalovirus (CMV) driven expression wanes over time, probably due to silencing of the promoter by hypermethylation (300). Transduction efficiency can be improved using woodchuck hepatitis post-transcriptional regulatory element (WPRE), which increases the efficiency of translation, and thus gene expression (298;301;302), and also chicken β-actin (CBA) promoter mediated gene expression is more efficient in the brain (286).

AIM AND OUTLINE OF THIS THESIS

As discussed above, the regulation of energy homeostasis involves a complicated neural circuitry, comprised of multiple brain areas and neuropeptides. Although much is known about the different neuropeptidergic systems in the hypothalamus, it is not fully understood what their specific role is in the diverse hypothalamic nuclei. The aim of the studies described in this thesis was to further elucidate the contribution of the different areas in the hypothalamus in the effects of the MC and NPY system on long-term regulation of energy balance.

To accomplish this, we have used viral gene transfer to obtain a local overexpression of agouti or NPY in several hypothalamic areas. The advantage of this approach is that injection of rAAV particles in the brain of an adult animal results in a stable long-term overexpression of the desired neuropeptide, thereby passing the compensational adaptations that occur when gene expression levels are altered during development. These features together (local, stable, long-term overexpression and normal development) are not feasible in common genetic or pharmacological strategies, where either the local or chronic component (pharmacological studies) or normal development (genetic studies) misses.

It is clear that reduced MC signalling, either by disruption of the MC4 receptor or by ubiquitous overexpression of the MC antagonist agouti, results in an obese phenotype. However, in these models signalling via the MC4R is already reduced throughout development, possibly resulting in the activation of counter regulatory systems. As described above, this can result in effects that may not be directly caused by the gene of interest. Moreover, gene expression of the MC4R or agouti in the entire brain and the periphery is altered, making it impossible to study the contribution of specific brain nuclei in MC signaling. The use of rAAV particles overcomes both these problems. In chapter 2 rAAV particles encoding agouti are injected in several hypothalamic areas to investigate their involvement of agouti-induced obesity in the adult rat, on both a normal and a high fat diet.

Opposite to increased agouti signalling, POMC overexpression results in a slightly decreased body weight gain, but normal food intake. Although it is hypothesized that the decrease in body weight gain is due to an overexpression of α-MSH, it is difficult to understand how this phenotype is established, since POMC encodes multiple melanocortin ligands as well as the endogenous opioid peptide β-endorphin that have variable effects on feeding behaviour. Chronic infusions of α-MSH are problematic, due to a short half-life of the peptide. In addition, relatively high concentrations are needed to elicit effects, due to a rather low affinity of α-MSH for the MC4R. Since it has been shown that multivalent ligands often have an increased affinity for their receptor (303), we have built rAAV viral vectors encoding multimers of α-MSH. In chapter 3, the construction, and the *in vitro* activity of these vectors is described.

NPY is one of the most potent orexigenic neuropeptides known. Acute NPY injections in the PVN induce hyperphagia and when these injections are repeatedly given for ten days animals get obese. However, the involvement of NPY signalling in the PVN on regulation of energy expenditure is not clear. Because it is not feasible to continuously infuse ligands in brain nuclei, we have used rAAV-NPY to clarify the role of chronically increased NPY signalling in the PVN in the development of obesity. This study is described in chapter 4.

Surprisingly, animals either lacking or overexpressing NPY since development show no obvious phenotype with regard to food intake and body weight. It is therefore hypothesized that counter-regulatory systems are activated during development that mask the effects observed after physiological-induced elevated or reduced NPY signalling in adult animals. To explore if chronic NPY overexpression that starts after development would also result in adaptations by other neuropeptidergic systems involved in energy balance we injected rAAV-NPY in the mediodorsal hypothalamus of young adult rats that were followed for 50 days. Furthermore, in this study it was also investigated by a pair-fed study to what extent the NPY-induced obesity was food intake-related. The results of this study are described in chapter 5.

The LH (including the PFA) is also reported to be involved in the effects of NPY on energy balance. With regard to food intake, the PFA is most responsive to acute injections of NPY, and injections into the LH are able to alter body temperature. In chapter 6 the specific role of the PVN and the LH/PFA associated with NPY-induced obesity are compared.

Altering concentrations of gene products by viral gene transfer is a relatively new strategy. In chapter 7, the pros and cons of this strategy are compared with more commonly used strategies in the exploration of energy balance.

Finally, in chapter 8 the main findings described in chapters 2-6 are summarized and discussed.