Understanding obesity by local overexpression of neuropeptides: a viral vector based approach

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Inzicht in obesitas door locale overexpressie van neuropeptiden:
een benadering gebaseerd op virale vectoren
(met een samenvatting in het Nederlands)

Proefschrift

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voor mijn vader

CONTENTS

ABBREVIATIONS

Acc	nucleus accumbens
ACTH	adrenocorticotropic hormone
AgRP	agouti-related protein
Arc	arcuate nucleus
BAT	brown adipose tissue
BDNF	brain-derived neurotropic factor
CART	cocaine and amphetamine-regulated transcript
CBA	chicken β-actin
CCK	cholecystokinin
CeA	central amygdala
CMV	cytomegalovirus
CNS	central nervous system
CRF	corticotropin-releasing factor
CTA	conditioned taste aversion
DMH	dorsomedial nucleus of the hypothalamus
DMV	dorsomotor nucleus of the vagus
DVC	dorsal vagal complex
GAL	galanin
GALP	galanin-like protein
HSPG	heparin sulfate proteoglycan
ICV	intracerebroventricular
ITR	inverse terminal repeat
LH	lateral nucleus of the hypothalamus
MC	melanocortin
MCH	melanin concentrating hormone
MCR	melanocortin receptor
MPO	medial preoptic area
MSH	melanocyte-stimulating hormone
NPY	neuropeptide Y
NSE	neuron specific enolase
NT	neurotensin
NTS	nucleus of the solitary tract
OX	orexin
OT	oxytocin
PB	parabracchial nucleus
PFA	perifornical area

POMC	proopiomelanocortin
PVN	paraventricular nucleus of the hypothalamus
PYY	peptide YY
rAAV	recombinant adeno-associated virus
RER	respiratory exchange ratio
SON	supraoptic nucleus
TRH	thyrotropin-releasing hormone
UCP	uncoupling protein
VMH	ventromedial nucleus of the hypothalamus
WAT	white adipose tissue
WPRE	woodchuck hepatitis post-transcriptional regulatory element
YR	NPY receptor

Chapter 1

General introduction

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).
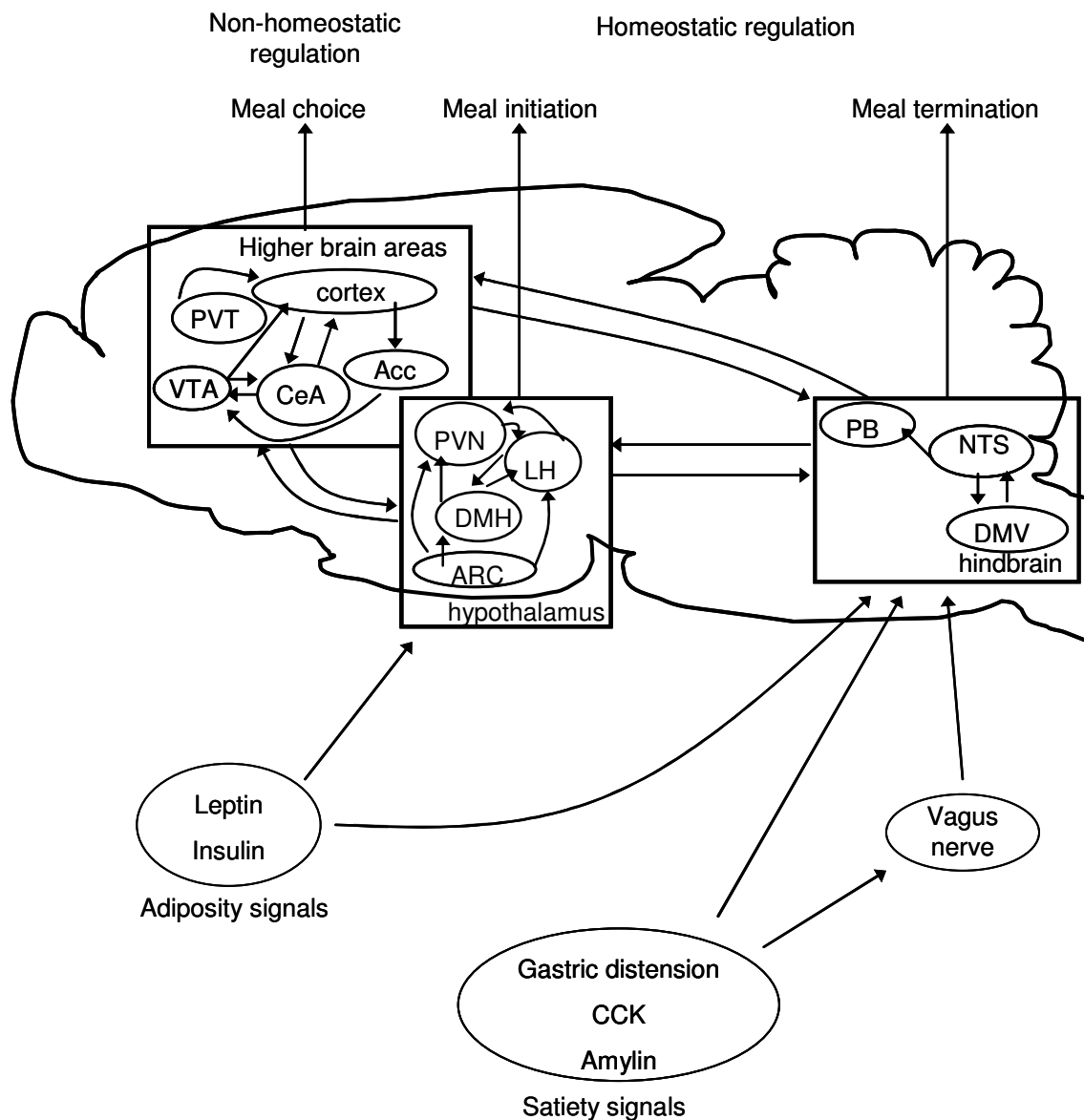

Figure 1: Schematic overview of the neural circuitry involved in the regulation of energy balance. Acc: nucleus accumbens, CeA: central amygdala, PVT: paraventricular nucleus of the thalamus, NTS: nucleus of the solitary tract, DMV: dorsomotor nucleus of the vagus, PB: parabracchial nucleus, VTA: ventral 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.
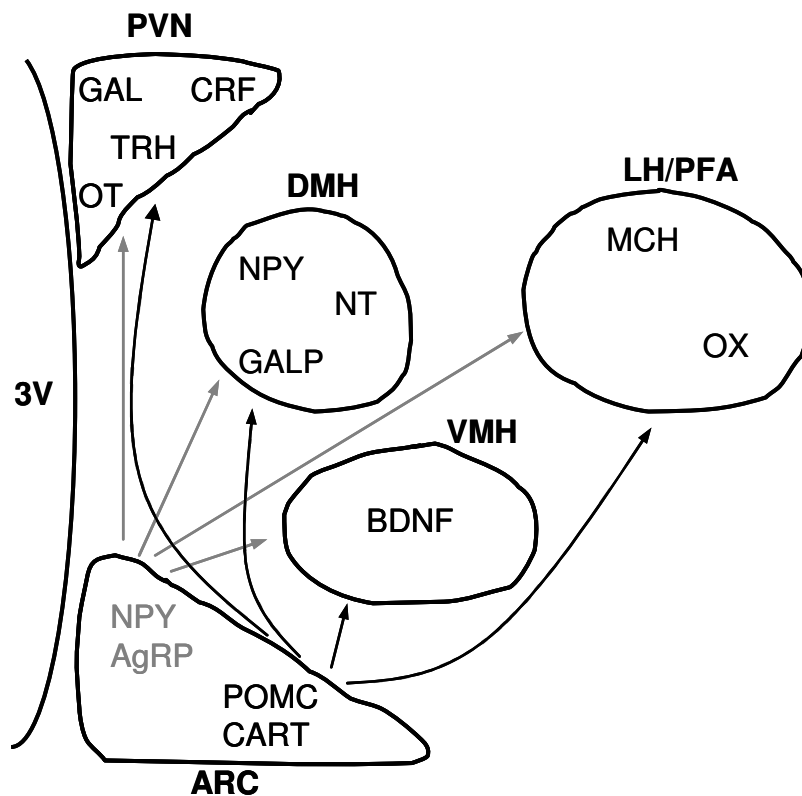
Figure 2: Schematic overview of the most important hypothalamic pathways and neuro-peptides involved in energy homeostasis. 3V: third ventricle, GAL: galanin, GALP: galanin-like protein, OX: orexin, OT: oxytocin, NT: neurotensin.

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 interpret, 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.

Chapter 2

Induction of brain-region specific forms of obesity by Agouti

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INDUCTION OF BRAIN-REGION SPECIFIC FORMS OF OBESITY BY AGOUTI

ABSTRACT

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

INTRODUCTION

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

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

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

MATERIAL AND METHODS

Recombinant adeno-associated viral vector production

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

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

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

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

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

Animals

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

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

Agouti antibody staining

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

In situ hybridization

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

Statistics

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

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

Ethical commission

Animal research and care was approved by the Ethical Commission on Laboratory Animal Experiments of the School of Medicine, University of Utrecht, The Netherlands.
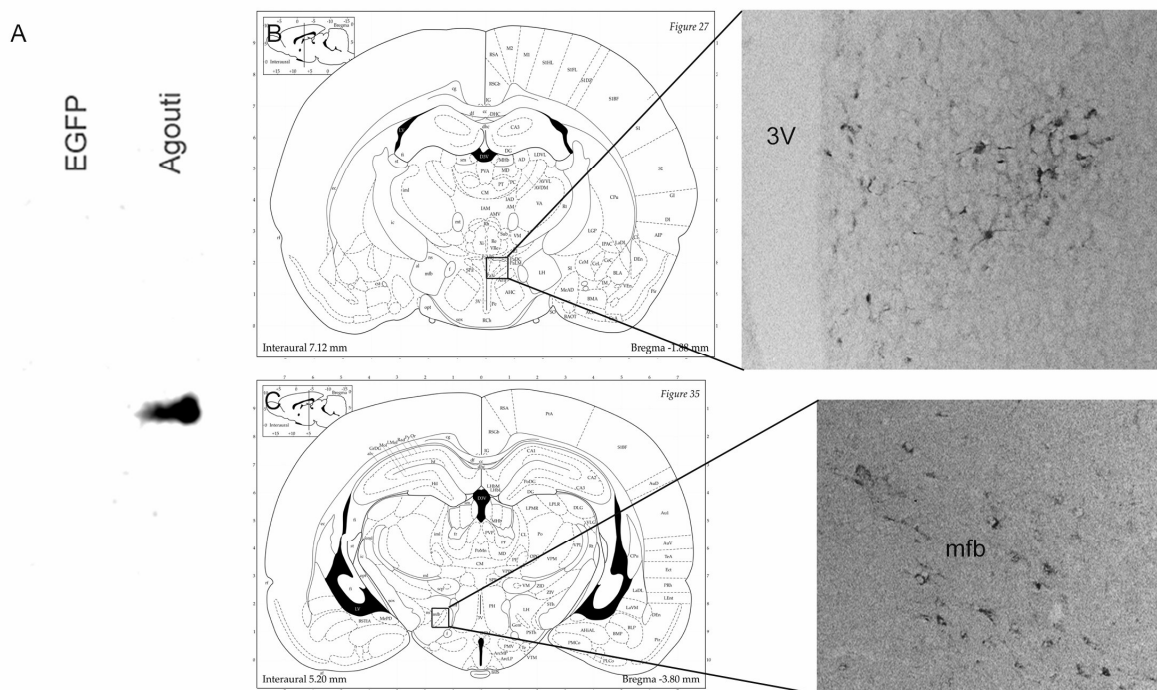

Figure 1: Agouti expression and secretion following infection with the rAAV-Agouti vector. Western blots (A) of media from HEK 293 cells that were infected with the AAV- EGFP-control (left lane) and AAV-Agouti (right lane) vectors were stained with an Agouti-antibody. No Agouti was observed in the media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

RESULTS

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

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

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

Unlike the rapid onset of obesity in PVN animals, onset was delayed when Agouti was expressed in the DMH. In the first three weeks following the injection, DMH-injected animals had similar levels of food intake and body weight when compared to controls. Thereafter, however, DMH-injected animals increased food intake and ate on average 2.5 grams per day more than controls. This resulted in a 30% increase in body weight gain (Figures 2E-F; p=0.0001). Since the DMH is located only 0.76 mm posterior from the PVN region involved in acute onset obesity, we investigated whether the delayed onset in DMH animals was a consequence of Agouti leakage to the PVN region. To exclude this possibility, we showed that rAAV-Agouti did not increase food intake and body weight when injected 0,76 mm anterior, instead of posterior, to the PVN region. To also exclude the possibility that the delayed onset of increased food intake and body weight resulted from a later start time
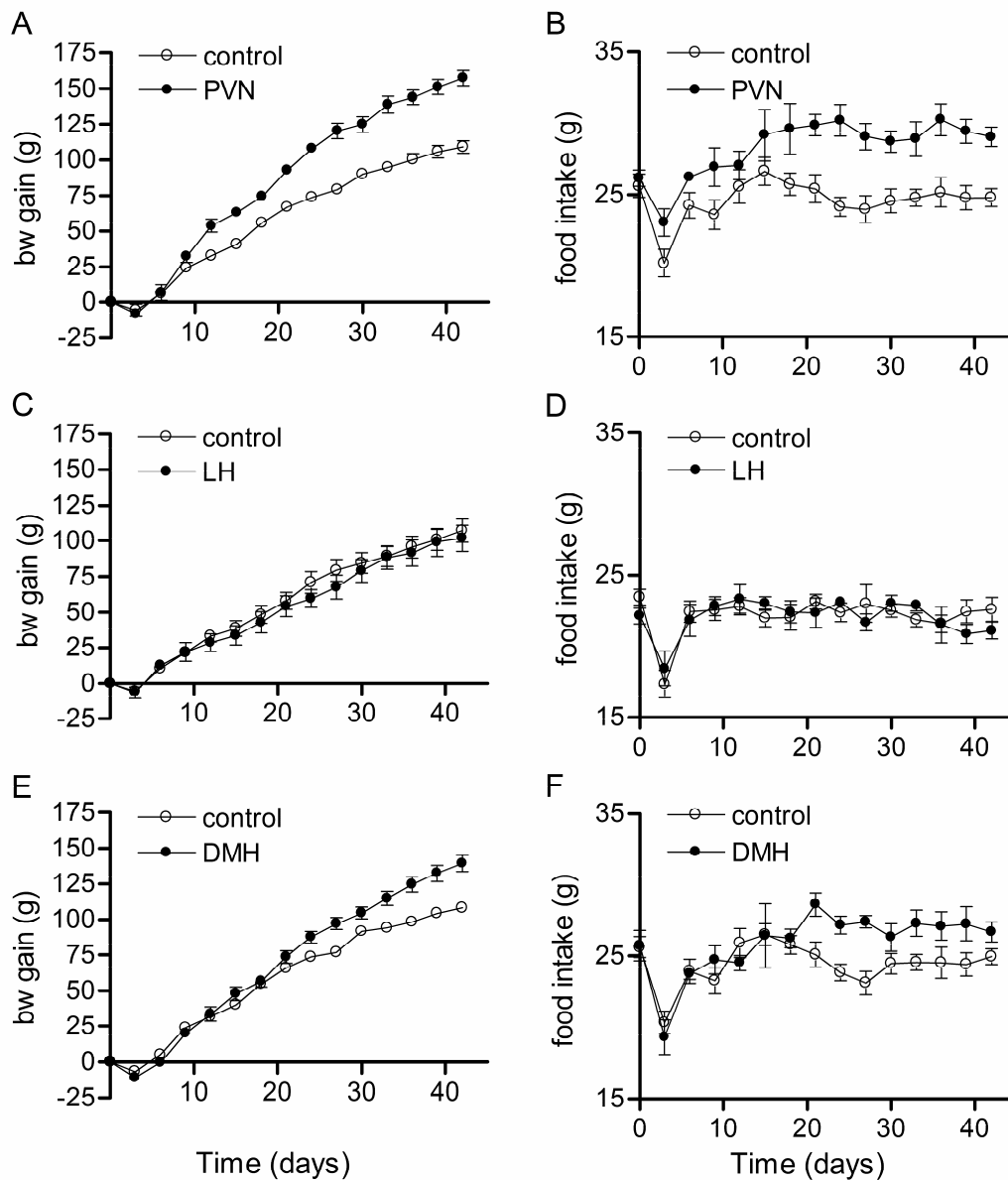
Figure 2: Agouti-expression in the PVN and DMH results in acute and delayed onset long-term hyperphagia-induced obesity, respectively. Body weight gain (A) and food intake (B) (both averaged over three days segments) are increased in animals with Agouti expression in the PVN when compared to EGFP-control PVN-injected animals. During *ad libitum* access to regular chow, body weight gain and food intake were similar in animals with Agouti expression in the LH and EGFP-control LH-injected animals (C and D). Agouti expression in the DMH the onset of increased body weight gain (E) and food intake (F) was delayed by three weeks, in contrast to the acute obesity onset in animals with Agouti expression in the PVN. In the first three days following surgery (days 0-3), a decline in body weight gain and food intake was observed in all animals. Histological verification of Agouti expression was checked for all animals by means of Agouti immuno-reactivity. Animals with Agouti positive cells in the targeted regions (PVN: -1.80 to -2.12 mm from bregma; LH: -2.56 to -3.80 mm from Bregma; DMH: -2.56 to -3.14 mm from Bregma, (region as defined by Paxinos and Watson, the rat brain in stereotaxic coordinates, 4th edition, 1998) were used for the analysis and resulted in the shown behavioural data-set for these confirmed groups (n=6-7 per group). * indicates p<0.0001.

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

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

Local long-term Agouti expression in the PVN, DMH and LH differentially induced hyperphagia and accelerated body weight gain without apparent changes in central neuropeptide systems that are known to stimulate food intake. We found that both AgRP and NPY mRNA levels in arcuate nucleus neurons, as well as MCH and orexin mRNA expression in the lateral hypothalamus were not affected following long-term Agouti expression in the PVN, DMH or LH (Figure 4).
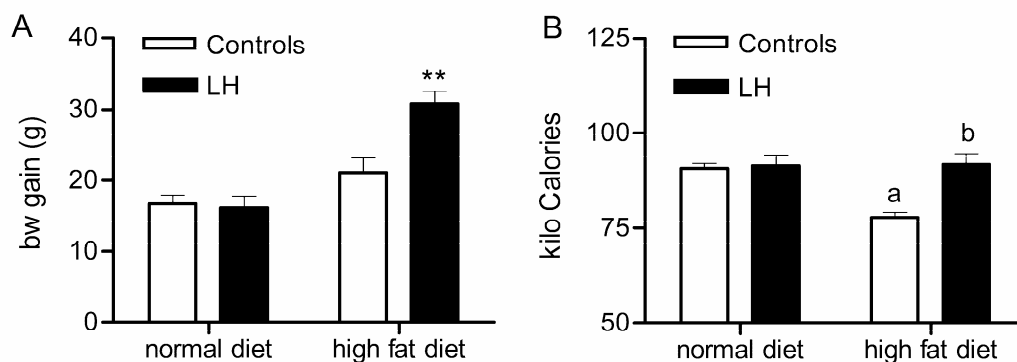

Figure 3: Agouti expression in the LH induced diet-induced obesity. While LH-EGFP control and LH-Agouti injected animals had similar levels of body weight gain during a normal diet (body weight gain during the last ten days of the normal diet), body weight gain significantly increased in animals with Agouti expression in the LH during ten days of access to a high fat diet (as compared to body weight on the day prior to the high fat diet) (A). This increased body weight gain in LH-Agouti injected animals related to increased caloric intake in LH-Agouti animals during the high fat diet (B) (** indicates different from all other conditions (p<0.0001); a indicates different from LH-EGFP during normal diet (P=0.006); b indicates different from LH-EGFP during high fat diet (P=0.003)).
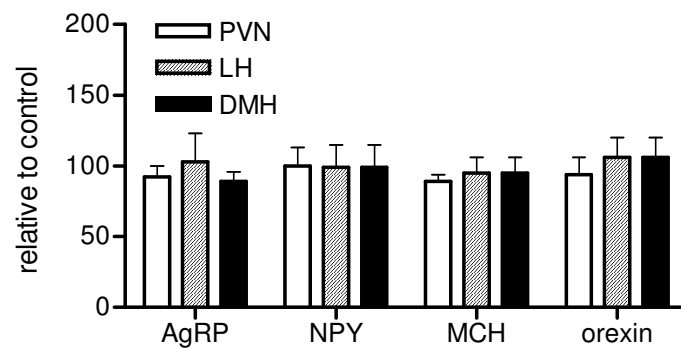
Figure 4: Hypothalamic mRNA expression levels of the orexigenic neuropeptides AgRP and NPY in the arcuate nucleus, as well as MCH and orexins in the lateral hypothalamus. Following long-term local expression of Agouti in the PVN, DMH and LH, no difference in mRNA expression levels were observed when compared to EGFP-controls (in brains of rats from Figures 2 and 3). mRNA expression levels are expressed as a percentage from the EGFP-controls. Since region specific EGFP-controls were not different in gene expression levels, their expression levels were pooled for the analysis.

DISCUSSION

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

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

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

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

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

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

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

Chapter 3

Multimeric α-MSH has increased efficacy to activate the MC4 receptor

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MULTIMERIC α-MSH HAS INCREASED EFFICACY TO ACTIVATE THE MC4 RECEPTOR

ABSTRACT

α-MSH has a relatively low affinity for the MC4 receptor. Constructs of multimeric α-MSH varying from one to eight subunits were synthesized to test whether they displayed an improved ability to bind to and activate the hMC4 receptor. α-MSH subunits were coupled by a flexible linker and placed in front of an IRES-EGFP sequence. Efficacy for activation of the MC4 receptor increased with every extra subunit, resulting in a 100 fold lower EC_{50} value of α-MSH8 when compared with α-MSH1. Furthermore, supernatant of cells transfected with α-MSH8 proved to have an increased affinity to the MC4 receptor when compared to cells transfected with the other multimers. Together, these data show that multimeric α-MSH has improved ability to activate the hMC4 receptor *in vitro*.

INTRODUCTION

Melanocortins (MCs) are a family of peptides derived from the precursor protein proopiomelanocortin (POMC). The MC system plays a key role in the central control of energy homeostasis and feeding. Situations where POMC expression is low, as in leptin- or leptin receptor-deficient mice, are associated with obesity. In addition, mutations in the POMC gene itself, in genes necessary for the processing of POMC and in the genes encoding the MC 3 or 4 receptor result in obese phenotypes (97;100;151;322;323). Mice that are POMC deficient are obese, due to hyperphagia, reduced basal oxygen consumption and an altered lipid metabolism (149-151). In line with this, transgenic overexpression of POMC under control of the NSE promoter in mice results in a slight decrease in body weight gain and also in reduced fasting-induced hyperphagia (131). Moreover, transgenic overexpression of (N-terminal) POMC or hypothalamic injection of recombinant adeno-associated virus (rAAV) encoding POMC ameliorates the obese phenotype of aged rats and genetically obese leptin- or leptin receptor-deficient mice and rats (131-133;324).

It is hypothesized that the ameliorating effects of POMC on obesity and metabolic function are mediated by the melanocortins α-MSH and β-MSH, products of POMC. Central α-MSH injections for 6 days in wildtype mice transiently reduce food intake and body weight (117). In addition, administration of an α-MSH analog reduces body weight of POMC^{-/-} mice (151). Also β-MSH inhibits food intake in fasted and non-fasted rats (86;87), and γ-MSH is, because it is a strong agonist for the MC3 receptor, also thought to play a role in the regulation of energy balance. Nevertheless, another cleavage product of POMC is β-endorphin, which in contrast to the melanocortins, increases food intake, making the control of energy homeostasis by POMC complicated (88).

In order to further clarify the specific role of MC receptors in energy balance it would therefore be necessary to chronically increase melanocortins, without altering concentrations of the other POMC products, in particular β-endorphin. Because the affinity of α-MSH for

the MC4 receptor is rather low and the half-life of α-MSH is relatively short, high concentrations of α-MSH are needed to elicit effects via the MC4 receptor. In addition, long-term infusions of a ligand locally in the brain are not feasible, making it difficult to explore the site-specific effects of α-MSH in the regulation of energy balance. To overcome this problem recombinant adeno-associated viral (rAAV) particles encoding α-MSH can be used, which, once injected into the brain, will result in a stable, long-term overexpression of the transgene (257).

Multivalant ligands often have increased binding affinity for their targets (303). Firstly, multivalent ligands increase the local concentration of binding elements, thereby increasing the chance the ligand will bind to its receptor. Secondly, subsequent binding to a second binding site of an oligomeric receptor can be facilitated when a ligand has more binding elements (avidity). Finally, multivalent ligands can promote receptor clustering, which can be necessary to activate signaling pathways (303). Melanocortin receptors have been proposed to occur as constitutively pre-formed dimers (325;326). In addition, there is evidence that the MC receptors have interacting binding sites and that they display by co-operative binding (325;327). Therefore, they form an excellent receptor type to target with multimeric ligands. Indeed, oligomers of NDP-MSH fragments were found to bind with a higher affinity to both the MC1 and the MC4 receptor than monomeric NDP-MSH (328;329).

To be able to study the contribution of a chronic α-MSH overexpression in distinct nuclei of the hypothalamus to melanocortinergic regulation of energy balance, we have built rAAV viral vectors encoding multimers of α-MSH cDNA. In this study, the synthesis of these multimers is described. Further, we evaluated the *in vitro* ability of these multimers to bind to and activate the MC4R.

MATERIAL AND METHODS

Cloning of α-MSH multimers

A signal sequence (belonging to the Von Willebrand factor), followed by an HA tag were synthesized using the following (partly overlapping) primers: forward1: 5'GGTGCTGCTTGCTCTGGCCCTCATTTTGCCAGGGACCCTTTGTTACCCCTACGACG3' , forward2: 5' TGTCCTCGAGGGCCATGATTCCTGCCAGATTTGCCGGGGTGCTGCTTGC TCTGG 3' and reverse1: 5' CATCTGAGCATGTCAAAATCTGGCCAGGCGTAGTCGGGCA CGTCGTAGGGGTAAC 3'. Primers were ligated, filled in using Klenow (USB Corporation, Cleveland, Ohio) and than cloned into pCRscript (Stratagene, La Jolla, California).

α-MSH monomeric cDNA was synthesized with primers (forward: 5'ACGCACCGG TCTCACCCCGCCTGGTTCTTCATCCTATTCCATGGAACACTTCAGGGGGGA 3' and reverse: 5'GAATTCACGTCTCCGGGGTGGAGGGTTTAGGCACAGGCTTTCCCCACCTG AAGTGTTCCAT 3') that are complementary to rat α-MSH cDNA including a flexible linker (Pro-Lys-Pro-Ser-Thr-Pro-Pro-Gly-Ser-Ser). Primers were ligated, filled in and than cloned

into pCRscript. Monomeric α-MSH was than cloned behind the signal peptide-HA-tag construct using AgeI and EcoRI. α-MSH multimers were synthesized by inserting BsaI/BsmBI digestion products of the monomer in a monomer linearized with BsmBI, resulting in α-MSH1, α-MSH2, α-MSH4 and α-MSH8 (Fig. 1A).

The monomer and multimers were than cloned into pIRES2-EGFP (USB Corporation, Cleveland, Ohio), using XhoI and EcoRI (Fig. 1B).
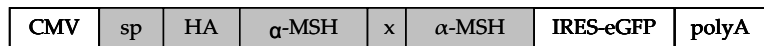
A

B

Figure 1: A. Nucleotide and aminoacid sequence of α-MSH2. Nucleotides and aminoacids in italic encode the signal peptide, nucleotides and aminoacids in italic and underlined encode the HA-tag, nucleotides and aminoacids in bold encode the linker sequence and nucleotides and aminoacids in bold and underlined encode an α-MSH subunit. In grey the recognition sites for BsaI and BsmBI are depicted. Interspaces depict the restriction sites and separation of the two α-MSH monomers.
B. Structure of the pIRES2-α-MSH2 multimer construct, containing a CMV promoter, the signal sequence of the Von Willebrand factor, an HA-tag and the α-MSH subunits, coupled by a flexible linker (x), followed by an IRES, EGFP and SV40 late polyadenylation signal (SV40 pA signal).

Cell culture and transfection

Human embryonal kidney (HEK)293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum (FCS, Integro, Zaandam, the Netherlands), 2 mM glutamine (Gibco, Paisley, Scotland) 100 units/ml penicillin, 100 units/ml streptomycin and non-essential aminoacids (NEAA, Gibco, Paisley, Scotland). Cells were cultured in a humidified atmosphere and 5% CO_2 at 37 Celsius.

DNA was transfected into cells with a standard calcium phosphate precipitation protocol. HEK293 cells cultured in 10 cm dishes were transfected with 10 µg of DNA of the α-MSH multimers or pIRES2. One day after transfection, medium was replaced by DMEM supplemented with 2% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 units/ml

streptomycin and non-essential aminoacids. Two days later, supernatant of cells transfected with the α-MSH multimers was harvested, concentrated 10 times using YM-3 Centricon filter units (Milipore, Billerica, Massachusetts) aliquotted and stored at -20°C until further use. For the reporter gene assay HEK293 cells cultured in 10 cm dishes were cotransfected with 50 ng human MC4R DNA and 10 μg of cAMP-Responsive Element (CRE)-LacZ construct. For the binding assay, the MC4 receptor was expressed in HEK293 cells by transfecting cells cultured in 10 cm dishes with 10 μg of DNA.

Western blot

For the preparation of cell lysates, cells transfected with the α-MSH multimers or pIRES2 were washed twice with PBS and incubated with M-PER protein extraction reagent (Pierce, Rockford, Illinois) for 5 minutes at RT. Cell debris was removed from the samples by centrifugation at 13,000 g for 5 minutes and supernatant was collected, aliquotted and stored at -20°C until further use.

Samples and standards (Kaleidoscope polypeptide standards, Bio-Rad laboratories, Hercules, Canada) were run on a 12% polyacrylamide gels using the tris tricine buffer system (330). Seperated proteins were transferred to nitrocellulose membranes (Hybond C, Amersham biosciences, Freiburg, Germany). Blots were blocked with 10% (w/v) non-fat milk powder, 0.05% Tween-20 in tris buffered saline (TBS) at RT while shaking and incubated overnight at 4°C with 1:1000 diluted mouse-anti-GFP antibody (Roche Diagnostics, Penzberg, Germany). Immunoreactivity was visualized using a peroxidase-conjugated secondary antibody and SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, USA) on a BioRad Fluor-S Multi-imager and analyzed with Quantity-One (BioRad, Tokyo, Japan).

Radioligand binding assay

IC_{50} values were determined by displacement of ^{125}I-[Nle^4, D-Phe^7]-MSH (NDP-MSH, PerkinElmer, Brussels, Belgium). Transfected HEK293 cells growing in 24-wells plates were washed with TBS supplemented with 2.5 mM calcium chloride and incubated for 30 minutes at RT with ^{125}I-NDP-MSH and various concentrations of the multimers diluted in HAM's F10 medium (Gibco, Paisley, Scotland) supplemented with 2.5 mM calcium chloride, 0.25% BSA (ICN, Aurora, USA) and 200 KIU/ml aprotinin (Sigma, Steinheim, Germany). Cells were rinsed twice with ice-cold TBS supplemented with 2.5 mM calcium chloride to remove non-bound tracer and lysed in 1M sodium hydroxide. Samples were than counted in a γ-counter.

Reporter gene assay

Activation of MC4 receptors was determined using LacZ as a reportergene (316). One day after transfection, cells were dispensed into 96-wells plates. After two days, cells were

incubated at 37 °C with α-MSH or with the concentrated supernatant containing the multimers diluted in half-LOG units in serum-free medium (DMEM containing 0.2% BSA (ICN, Aurora, USA). After 5-6 hours, the assay medium was aspirated and replaced by 40 µl of lysis buffer (PBS containing 0.1% Triton-X-100 (Boehringer, Mannheim, Germany)). The plates were stored at -20 °C and after thawing 80 µl of substrate mix (0.1 M phosphate buffer, pH7.4 containing 1.6 g/l o-Nitrophenyl β-Dgalactopyranoside (ONPG, Molecular probes, Leiden, the Netherlands), 67.5 mM β-mercaptoethanol (Merck, Darmstadt, Germany) and 1.5 mM magnesium chloride) was added. Absorbance at 405 nm was measured in a Victor² microplate reader (PerkinElmer, Brussels, Belgium).

Data analysis

Data of the binding assay and the reporter assay were analyzed using GraphPad Prism (GraphPad Software Inc, San Diego, California). Competition curves were fitted from 6 duplicate data points (12 for α-MSH) using the sigmoidal dose-response curve (variable slope) classical equation for non-linear regression analysis and IC_{50} values were calculated. Differences in maximal displacement data were assessed using one-way ANOVA, followed by Tukey's post-hoc tests. In the reporter assay for each curve 12 duplicate data points were collected and EC_{50} values were determined by fitting the data to a sigmoidal dose-response curve with variable slope.

RESULTS

Transfection of HEK293 cells with α-MSH-multimers

HEK293 cells were transfected with the different α-MSH multimers or pIRES2-EGFP. Supernatant was collected and used in a binding assay and a reporter gene assay. The cells were lysed and analyzed for EGFP expression. Figure 2 shows a western blot of the cell lysates of the cells transfected with the α-MSH multimers. Cell lysates of cells transfected with the multimers contained a single band that ran at the same height as the EGFP band from cell lysate of the cells transfected with pIRES2-EGFP. All the multimers showed a similar expression of EGFP, which was higher than the EGFP expression of pIRES2-EGFP. This indicates that similar amounts of vector were introduced in cells expressing the multimers.
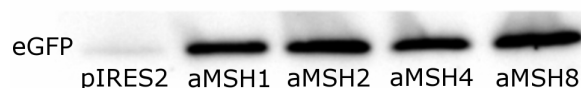

Figure 2: Western blot of cell lysates of HEK-293 cells transfected with pIRES2 or α-MSH multimers and stained using an EGFP antibody.

Binding properties of multimeric α-MSH

Competition binding showed that supernatant from cells transfected with α-MSH1, α-MSH2 and α-MSH4 displayed similar (low) binding properties to the hMC4 receptor, whereas supernatant from cells transfected with α-MSH8 had a slightly higher affinity (data not shown). No IC50 values for α-MSH1, α-MSH2 and α-MSH4 could be calculated, since the highest concentration of supernatant containing these multimers was not able to reach maximal displacement. Unfortunately, it was technically not feasible to further increase the concentration of multimers without non-specifically interfering with the binding assay. The IC50 value for α-MSH8 is presented in table 1. The percentage of displacement reached by a two-fold dilution of supernatants is presented in figure 3, and was analyzed using one-way ANOVA. This revealed that displacement properties of α-MSH1 were not significantly greater than pIRES2 supernatant. All the other multimers however did show improved displacement when compared to pIRES2 or α-MSH1 supernatant ($p<0.05$). Furthermore, supernatant containing the highest concentration of α-MSH8 was able to displace most ^{125}I-NDP-MSH ($p<0.001$ compared to all other supernatants).

Table 1: IC_{50} values and EC_{50} values of the α-MSH-multimers. IC_{50} values were obtained by displacement of iodinated NDP-MSH. EC_{50} values were determined in a *LacZ* reporter gene assay. IC_{50} values and EC_{50} values are given as LOG values of the dilution of the supernatant. nd: not determined.

Multimer	Binding IC_{50} (LOG dilution)	Activation EC_{50} (LOG dilution)
α-MSH1	nd	-0.9439
α-MSH2	nd	-2.015
α-MSH4	nd	-2.310
α-MSH8	-1.026	-3.024

MC4 receptor activation

Dose response curves of all α-MSH multimers were obtained and efficacy for activation of the hMC4 receptor was compared with α-MSH. Whereas supernatant of HEK293 cells transfected with pIRES2-EGFP was not able to activate the hMC4 receptor, all α-MSH multimers were capable to activate the hMC4 receptor (Fig. 4). Moreover, the highest concentration that we were able to test of all α-MSH multimers except α-MSH1 was capable to activate the hMC4 receptor to the same extent as the highest concentration of α-MSH. However, with the addition of every α-MSH subunit, the EC_{50} value of the multimer decreased (Table 1), resulting in a 100 fold higher affinity of α-MSH8 when compared to α-MSH1.

Furthermore, activation induced by a 100 fold dilution of the concentration that resulted in 20% displacement in the binding assay gradually increased with each subunit (Fig. 5).
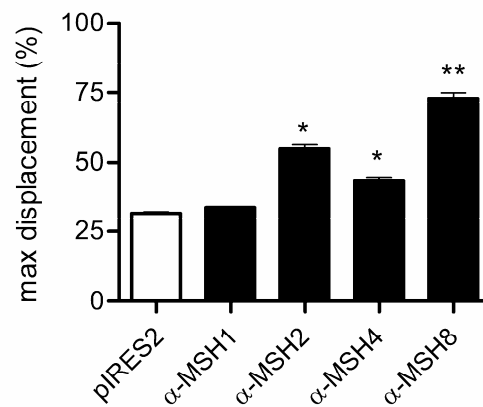
Figure 3: Displacement of hMC4 receptor bound ^{125}I-NDP-MSH by a 2-fold dilution of concentrated supernatant of cells transfected with the multimers. *$p<0.05$ vs pIRES2 and α-MSH1, **$p<0.01$ vs all other multimers.
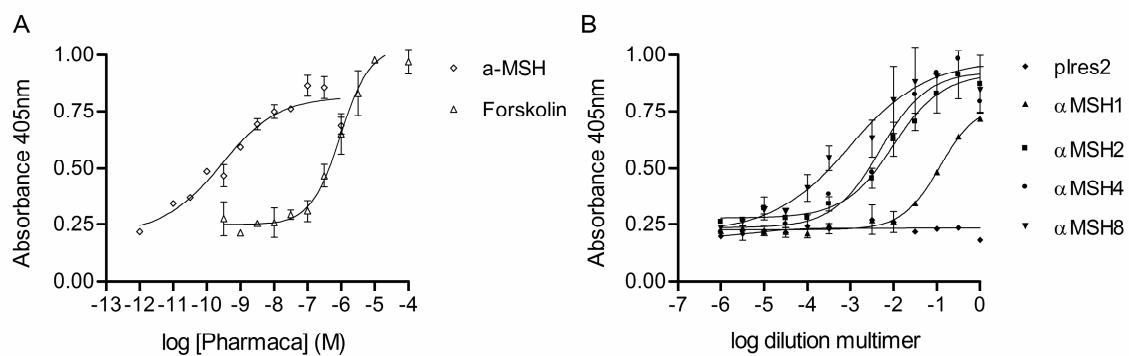

Figure 4: Dose-response curves of hMC4 receptor activation by α-MSH, forskolin (A) or α-MSH multimers (B), measured by CreLacZ reporter gene assay.

DISCUSSION

The present study described the synthesis of multimeric forms of α-MSH, and their ability to bind to and activate the hMC4 receptor. We have shown that with the addition of each α-MSH subunit, the affinity of the multimer for the MC4 receptor increases. Interestingly, when similar dilutions of concentrations of multimers that gave equal displacement in a binding assay were tested for activity, constructs with more α-MSH subunits were clearly more effective than expected based upon binding. This indicates that, independent from the affinity for the MC4 receptor, also the ability to activate signaling pathways downstream the MC4 receptor increased with each extra subunit.

Oligomerization of ligands can increase their affinity. For NDP-MSH fragments, affinity for the MC4 receptor has been reported to increase stepwise from monomer to trimer (329). The length and structure of a linker can influence the binding affinity of

oligomers (331). Dimers separated by an ideal length can display an affinity of 150 times higher than the monomer (331). To prevent the linker to interfere with the binding of a ligand to its receptor, the linker must be hydrophilic and small (329). Indeed, multimers of NDP-MSH fragments with various short linkers have been shown to have an increased affinity for the MC1 and MC4 receptor (328;329). With the linker length, also the IC50 value of the multimer increased (329). In addition, the linker should not be too flexible, because this could increase the entropic cost to bind to a second binding site, thereby diminishing the avidity of the multimer (329). Vagner et al showed that linkers of the same length, but with variations in flexibility have different effects on the improved binding capabilities of NDP-MSH fragment oligomers (329).
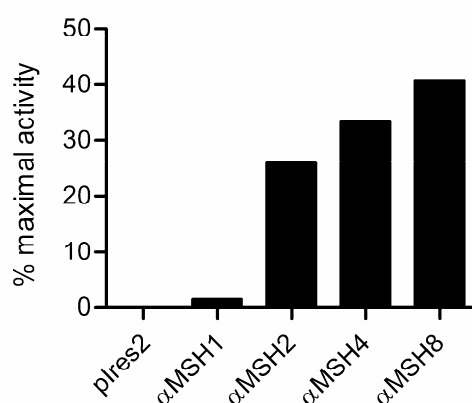

Figure 5: Activation of hMC4 receptors at a 100 fold dilution of the concentration that results in 20% displacement of ^{125}I-NDP-MSH, as percentage of maximal activity.

Our results indicate that also multimers of full length α-MSH, coupled by a relatively long, flexible linker can increase the affinity to bind to the MC4 receptor, as shown by the competitive binding assay. Cells transfected with α-MSH8 had a lower IC_{50} value compared to the rest of the multimers. In addition, α-MSH8 was able to displace significantly more ^{125}I-NDP-MSH from the hMC4 receptor than a similar concentration of α-MSH4 and α-MSH2, which, on their part, were more capable in displacing ^{125}I-NDP-MSH than α-MSH1.

Furthermore, also the capability to activate signaling pathways downstream the MC4 receptor was increased, which was at least partly independent from the increased binding properties. Whereas the highest concentration of α-MSH8 that we tested was only able to displace 2 times as much ^{125}I-NDP-MSH from the hMC4 receptor than α-MSH1, the EC_{50} value had decreased 100 times. It is known that activation of the MC4 receptor by its agonist *in vitro* is associated with time-dependent and concentration-dependent internalization (332;333). Reduced internalization of the MC4 receptor due to binding to multiple subunits of an α-MSH multimer could very well explain the increased activation properties of α-MSH8 compared to the binding affinities

Despite the fact that rigid linkers have been proven to result in the highest increase in affinity, rAAV vector-derived multimers are built up from aminoacids, which are flexible. Even though flexible linkers can decrease avidity, due to loss of entropy, they still increase the local concentration of ligand. Furthermore, they may still promote clustering of receptors. Based upon rhodopsin, which is the only G-protein-coupled receptor (GPCR) for which the crystal structure is available, the distance between the centers of the binding sites of dimeric GPCRs is about 38 Å (334). The distance between two α-MSH subunits in our multimers in the maximal extended form is 70.4 Å, which is therefore enough for a multimer to bind simultaneously to both binding sites of a MC4 dimer. Since MC receptors display by co-operative binding (325;327), these properties will still favor multimeric αMSH over the monomer. Although short, rigid linkers may be the best solution for fragmental ligands, for full length α-MSH, which represents the best physiological situation, a flexible linker may be better to provide the folding necessary to bind a receptor dimer simultaneously.

α-MSH has a relatively low affinity for the MC4 receptor. Nevertheless, the weight reducing effects of POMC overexpression are ascribed to the actions of α-MSH (117;151). However, besides melanocortins, also other cleavage products of POMC, especially β-endorphin, can affect energy balance when injected into the brain (88). Combining the increased receptor binding and activation properties of the α-MSH multimers described in this study with the long-term local overexpression of rAAV-delivered transgenes would be an excellent tool to increase the knowledge of site-specific increased melanocortin signaling in energy balance.

Chapter 4

Injection of rAAV-NPY in the paraventricular nucleus results in obesity

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INJECTION OF RAAV-NPY IN THE PARAVENTRICULAR NUCLEUS RESULTS IN OBESITY

ABSTRACT

Chronic central administration of NPY has dramatic effects on energy balance, however, the exact role of the hypothalamic paraventricular nucleus (PVN) in this is unknown. The aim of this study was to further unravel the contribution of NPY signaling in the PVN to energy balance. Recombinant adeno-associated viral particles containing NPY (rAAV-NPY) were injected in the rat brain with coordinates targeted at the PVN. For three weeks, body weight, food intake, endocrine parameters, body temperature and locomotor activity were measured. Furthermore, effects on insulin sensitivity and expression of NPY, AgRP and POMC in the arcuate nucleus were studied. Food intake was increased specifically in the light period. Furthermore, dark phase body temperature and locomotor activity were reduced. This resulted in obesity characterized by increased fat mass, elevated plasma insulin, leptin and adiponectin, decreased AgRP expression in the arcuate nucleus and decreased insulin sensitivity, whereas plasma corticosterone was unaffected. These data suggest that increased NPY expression targeted at the PVN is sufficient to induce obesity. Interestingly, plasma concentrations of leptin and insulin were elevated prior to a rise in food intake, suggesting that NPY in the PVN influences leptin and insulin secretion independent from food intake. This strengthens the role of the PVN in regulation of energy balance by NPY.

INTRODUCTION

Neuropeptide Y (NPY), which is widely distributed throughout the brain, has a robust feeding effect when injected centrally (193;220). Moreover, it decreases metabolic rate and thermogenesis (206;208;335). One week of chronic intracerebroventricular (ICV) administration of NPY results in an obese phenotype characterized by hyperphagia, elevated plasma concentrations of corticosterone, leptin and insulin, and increased lipogenesis in liver and adipose tissue (121;201;203;212;214).

Neurons in the arcuate nucleus that express NPY have been implicated in the regulation of energy balance and project to a variety of hypothalamic nuclei, including the paraventricular (PVN), dorsomedial (DMH) and lateral (LH) hypothalamic nuclei and the medial preoptic area (56;177-179). Although all these nuclei may be involved in this regulation, acute injections of NPY in the PVN and the perifornical area result in the largest increase in food intake (217;223). Furthermore, repeated daily injections of NPY in the PVN of female rats result in increased (high fat) food intake and body weight, however whether this increased weight gain is solely due to food intake or also to decreased energy expenditure remains to be determined (225). The role of NPY in the PVN for thermogenesis is contradictory; Currie *et al.* found a hypothermic effect within 3 hours after NPY injection in the PVN (223), Jolicoeur *et al.* found the opposite (205). To further unravel the role of NPY in the PVN in the regulation of energy balance, infusion of NPY in the PVN over longer

periods is required. It is, however not feasible to reliably infuse ligands locally in the brain for weeks.

One way of approaching this problem is genetic overexpression of NPY. However, results obtained using genetic overexpression of NPY are not consistent with data from pharmacological NPY administration. Global overexpression of NPY in transgenic mouse and rat models results in normal body weight and food intake when fed a regular chow diet (230;231;233). This indicates that in these transgenic models there is either a developmental change, compensation by counter-regulatory mechanisms, or only limited overexpression. By ablation of NPY/AgRP neurons, it was shown recently that, although these neurons are necessary for normal feeding in adult mice, they can be ablated without affecting body weight gain or food intake in neonates (155;156). Furthermore, a 4-fold NPY overexpression of NPY did not affect body weight regulation (232), suggesting an enormous plasticity of these systems during development.

Taken together, there are discrepancies between the strong effects after chronic icv administration of NPY and the modest effects of transgenic NPY overexpression on food intake and body weight. Furthermore, the specific involvement of the PVN in the obese phenotype is not clear. In order to bypass developmental compensation and address the role of long-term NPY signaling specifically in the PVN in the regulation of energy balance, we used viral gene transfer. Recombinant adeno-associated viral (rAAV) particles containing NPY cDNA were injected to induce local overexpression of NPY in the PVN, that is still present three weeks after injection. Rather than overexpressing NPY in the Arc, being the major source of NPY released in the PVN, we injected AAV-NPY directly in the PVN. A feature of AAV2 transduction in the brain is that the vector remains confined to the injection site (336). Indeed our earlier findings, using a similar strategy to overexpress agouti, showed that local immunostaining was limited to the PVN, and not observed in projection areas of the PVN (337). Thus, we and others (338) have demonstrated that AAV-mediated overexpression is suitable to induce local delivery of gene products. The effect of long-term overexpression of NPY in the PVN was evaluated by daily recording of body weight and food intake, and repeated analysis of hormonal changes and glucose tolerance. Finally, we also measured body temperature and locomotor activity.

MATERIAL AND METHODS

Animals

Male Wistar rats weighing 220-250 g were purchased from Charles River (Crl-Wu, Germany). They were individually housed in filtertop cages with *ad libitum* access to food and water. Animals were kept in a temperature and humidity controlled room (21±2 °C) under a 12h/12h light/dark cycle (lights on at 0700 h). All experimental procedures were

approved by the Committee for Animal Experimentation of the University of Utrecht, Utrecht, The Netherlands.

Surgical procedures

Rats were anesthetized with 0.1 ml/100 g im fentanyl/fluanisone (Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium) and 0.05 ml/100 g ip midazolam (Dormicum®, Hoffman-LaRoche, Mijdrecht, The Netherlands). A silicone catheter was implanted in the right jugular vein and attached behind the shoulders with a backmount pedestal (Bilaney Consultants, Dusseldorf, Germany). Transmitters (TA10TA-F40 Data Science International, St Paul, Minnesota, USA) were placed in the abdominal cavity. Rats were left to recover for three weeks.

Seven days after baseline recordings, rats were anesthetized again as described above. Using a stereotax, rAAV-NSE-NPY (n=10) or rAAV-NSE-empty (used as control) (n=6) was injected bilaterally into the PVN (rAAV-NPY-PVN and rAAV-contr-PVN, coordinates AP: -1.8 mm from bregma, ML: ±0.3 mm from bregma, and DV: -8.0 mm below the skull). Another control group was injected bilaterally with rAAV-NSE-NPY into the thalamus (rAAV-NPY-thal, coordinates AP: -1.8 mm from bregma, ML: ±0.5 mm from bregma, and DV: -7.0 mm below the skull) (n=5). Production of rAAV-NPY has been described previously (339;340). 1 µl of virus ($2x10^8$ particles) was injected per site over five minutes, after which the needle was kept in place for ten minutes before removal.

After each surgery, rats received an injection with 0.05 ml/100 g sc buprenorphin (Temgesic®, Schering-Plough, Maarssen, The Netherlands).

Data analysis

One week before, till three weeks after viral injections, body weight, food intake, body temperature and locomotor activity were recorded. Body weight gain and food intake were measured daily at 1400 h. Feed efficiency (FE) of the rats was calculated as the ratio of body weight gain over four days and the total food intake of that period (day 8-11 and day 18-21).

Locomotor activity and body temperature were measured via the transmitters that send digitized data via radio frequency signals to a nearby receiver. The data were automatically recorded every ten minutes, and averaged per hour using DSI software (DSI, St Paul, MN).

Naso-anal length and waist circumference were measured at day 22 in awake animals.

Glucose tolerance test

An intravenous glucose tolerance test was performed at day 17 after injection of the AAV, between 1000h and 1100h. Rats were fasted for 1 hour and injected with glucose (150 mg in 0.5ml saline) via the jugular vein cannula, followed by 0.5 ml saline. 200 µl blood samples were taken via the jugular vein cannula just before, and 1, 5, 10, 20, 30 and 45 minutes after glucose administration. Blood glucose and plasma insulin were measured as described under plasma analysis.

Collection of blood and tissues

Blood samples were collected at ± 1000 h at seven and fourteen days after injection. A PE tube was connected to the backmount pedestal, catheters were rinsed with heparinized saline, blood samples of 2 ml were taken and collected in tubes containing 0.1 M EDTA, after which rats received 2 ml of saline. At day 23 after injection, rats were decapitated, trunk blood was collected in heparinized tubes after adding 83 µmol EDTA and 1 mg aprotinin, and immediately placed on ice. Plasma samples were stored at -20 °C until further analysis.

Brains were immediately removed after decapitation, quickly frozen in cold isopentane (-35 °C) and stored at -80 °C. Retroperitoneal and epididymal white adipose tissue (WAT), interscapular brown adipose tissue (BAT), liver and adrenals were isolated and weighed.

Localization of rAAV in the brain

16 µm coronal sections of the hypothalamus were sliced using a cryostat (Leica, Rijswijk, The Netherlands), thaw-mounted onto RNAse free Superfrost slides (Menzel, Germany) and stored at -80 °C. Viral infection was localized by in situ hybridization (ISH) with a digoxigenin (DIG)-labeled woodchuck post-transcriptional regulatory element (WPRE) probe. The WPRE sequence is part of the expression cassette of all vectors used here. ISH procedure has previously been described (341).

Immunohistochemistry

16 µm coronal sections of the hypothalamus were fixated in 4% paraformaldehyde. After pretreatment with 0.3% H_2O_2, slides were incubated overnight at 4 °C with a rabbit anti-NPY antibody (Niepke, 1:2000, a kind gift of Prof. Dr R.M. Buijs, Amsterdam, the Netherlands). Sections were then incubated for one hour with a secondary goat anti-rabbit-biotinylated antibody (1:100) (Jackson ImmunoResearch). After incubation for one hour with ABC (1:500) (Vector Laboratories, Burlingame, California, USA) sections were treated with diaminobenzidine (Sigma, Saint Louis, Missouri, USA, 0.5 mg/ml in tris buffered saline, with 30% H_2O_2) for ten minutes. All immunohistochemistry steps described above were followed

by a three times rinse with tris-buffered saline. Slides were then dehydrated in ethanol, cleared with xylene and coverslipped.

Plasma analysis

Plasma leptin, insulin, adiponectin and corticosterone were analyzed in duplicate using radioimmunoassay kits, (Linco Research, St Charles Missouri, USA for leptin, insulin and adiponectin, ICN Biochemicals, Costa Mesa, California, USA for corticosterone). Plasma adrenocorticotropic hormone (ACTH) was measured in duplicate using a specific rabbit antiserum directed to the midportion of ACTH, which was kindly provided by Dr G.B. Makara (Budapest, Hungary). Synthetic human $ACTH_{(1-39)}$ (Peninsula Laboratories, Belmont, California, USA) was labeled with ^{125}I and used as tracer (342). Plasma glucose was measured in duplicate using a Medisense glucosesensor (Abbott, Amersfoort, The Netherlands). Plasma free fatty acids (FFA), triglycerides (TG) and glycerol were measured in triplicate using an Acyl-CoA synthetase-acyl-CoA oxidase method (FFA, Roche Diagnostics, Penzberg, Germany) or a serum triglyceride determination kit (TG and glycerol, Sigma, Saint Louis, Missouri, USA).

Quantitative in situ hybridization

16 µm coronal sections were used for raISH. 33P-labeled antisense RNA probes were made using AgRP (396 bp mouse (343)), NPY (286 bp rat (344)) and POMC (350 bp rat (343)) cDNA fragments. raISH procedure has previously been described (345). Expression of AgRP, NPY and POMC was analyzed in the arcuate nucleus using Image J software (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

Data are presented as group means ± SEM. Differences in body weight gain, food intake, body composition and plasma levels were assessed using one-way ANOVA, followed by Tukey's post-hoc test. Body temperature and locomotor activity were analyzed by T-tests. Differences were being considered significant at $p < 0.05$.

RESULTS

Localization of rAAV in the brain

The location of microinjection of rAAV vectors in PVN or thalamus (which served as a control group) was confirmed by post mortem WPRE *in situ* hybridization (Fig. 1). For rAAV-NPY-PVN and rAAV-contr-PVN, rats were only included in the study when WPRE expression was bilaterally in the PVN (Fig. 1A). In some of the rats that were included in these groups there was also staining of cells in the periventricular nucleus and in the thalamus surrounding the injection shaft. Three rats, where the PVN was hit unilaterally,

and/or only partly, were excluded from the rAAV-NPY-PVN group and were considered separately as controls (i.e. indicated as *missed* in Fig. 2) . From rAAV-NPY-thal rats (Fig. 1B), only one rat was excluded, because no WPRE expression was found. We found no WPRE positive cells in any hypothalamic nucleus in any of the rats included in this group.

At the end of the study (day 23), punctate NPY staining was observed in the thalamus of the rAAV-NPY-thal rats, as well as NPY-positive cell bodies. In rAAV-NPY-PVN rats, total NPY staining in the PVN, but not outside the injection area, was increased when compared to controls, as shown by NPY immunohistochemistry (Fig. 1C).
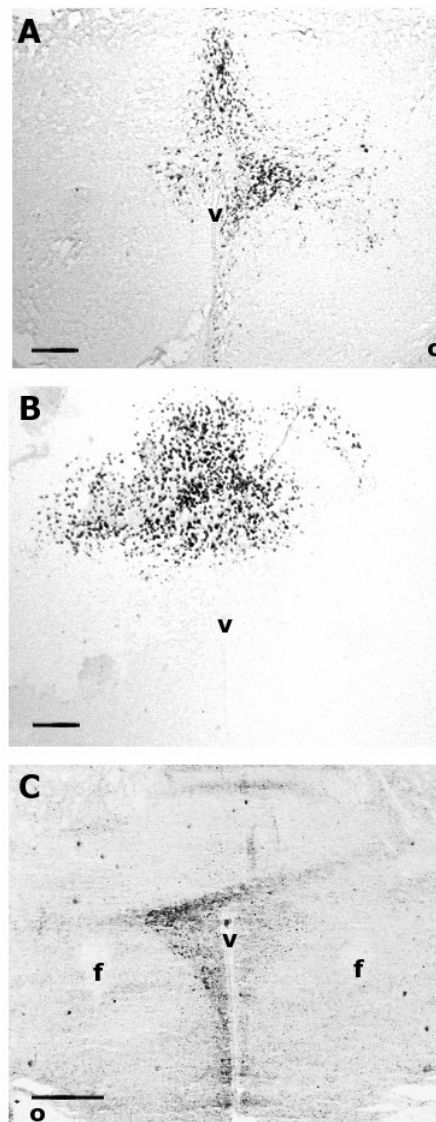

Figure 1: Localization of WPRE RNA expression in hypothalamic (A) and thalamic (B) sites injected with rAAV-NPY. (C) Photomicrograph of immunohistochemistry for NPY in the PVN in a rat injected unilaterally (left side) in the PVN with rAAV-NPY. Note the increased staining at the infection side. f = fornix, o = optic tract, v = third ventricle. Scalebar = 500 μm.

Effects of rAAV-NPY on body weight and food intake

The first three days after injection, there was a similar decrease in body weight and food intake in all groups of rats. Seven days after injection, all rats had recovered their initial body weight and food intake.

Food intake increased in rAAV-NPY-PVN rats and reached a plateau at fifteen days after injection (Fig. 2A). In the last week of the experiment, rAAV-NPY-PVN rats ate over 75% more food as compared to rAAV-contr-PVN and rAAV-NPY-thal rats. Food intake of the 'missed' injection group of rats was intermediate between that of rAAV-NPY-PVN 'hit' rats and controls.

Interestingly, food intake of rAAV-NPY-PVN animals was increased in the light phase, but not in the dark phase. Light phase feeding increased over time in rAAV-NPY-PVN 'hit' rats, but was stable in controls (Fig. 2B).
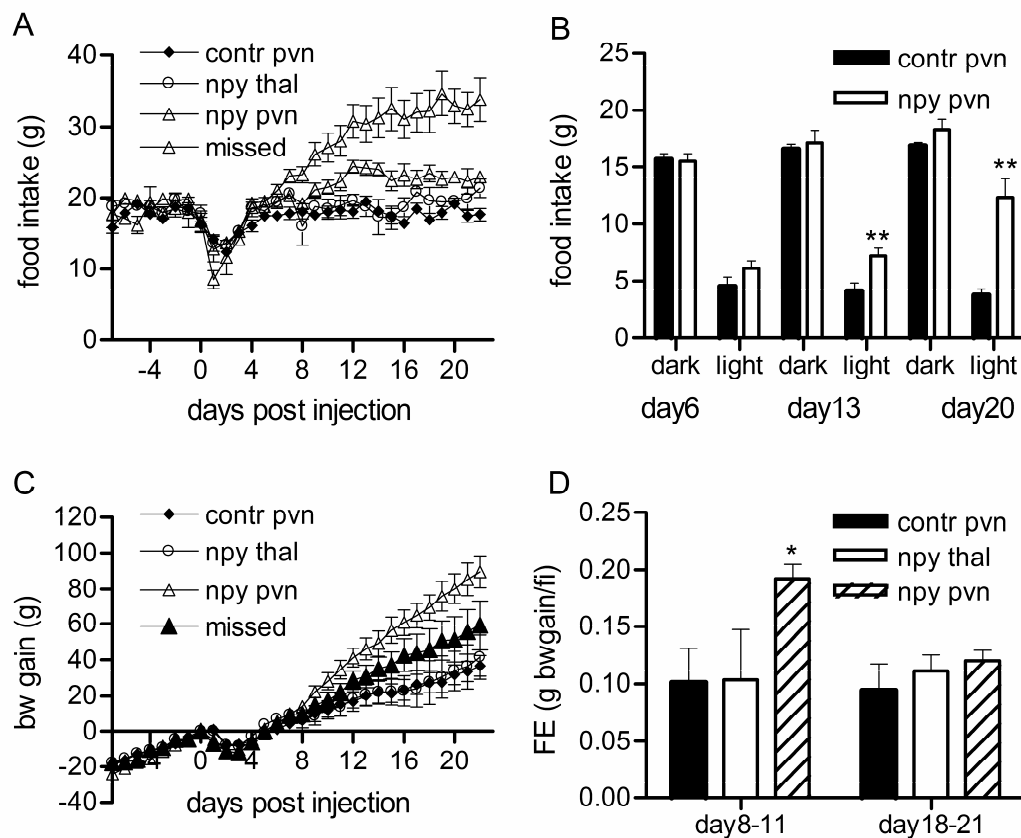

Figure 2: Effects of rAAV-NPY injections on daily food intake (A and B) and cumulative body weight gain (C) compared to rAAV-contr-PVN rats, rAAV-NPY-thal rats, and rats of the 'missed' injection group. D. The body weight gain per grams of food intake (Feed efficiency (FE)) calculated between day 8-11 and 18-21 after rAAV injection. * $p < 0.05$, ** $p < 0.01$.

Whereas rAAV-contr-PVN and rAAV-NPY-thal rats showed a slow gain of body weight after having recovered from the injection, similar to body weight gain before injection, rAAV-NPY-PVN rats showed a strong increase in body weight gain, starting at day 9 following injection (Fig. 2C). This led to an accumulated body weight gain of 240% compared to rAAV-contr-PVN rats on day 22. Body weight gain of the 'missed' injection group of rats was intermediate between rAAV-NPY-PVN rats and control groups.

To determine whether the body weight increase observed in rAAV-NPY-PVN rats was only due to food intake changes, we measured feed efficiency (FE), calculated as the ratio of body weight gain per total food intake over four days in individual rats. Since the food intake curve (Fig. 2A) was increasing until day 15 and was stable afterwards, these two phases were analysed separately. FE was significantly increased between day 8-11 after injection; however, between day 18-21 there was no difference anymore in FE (Fig. 2D).

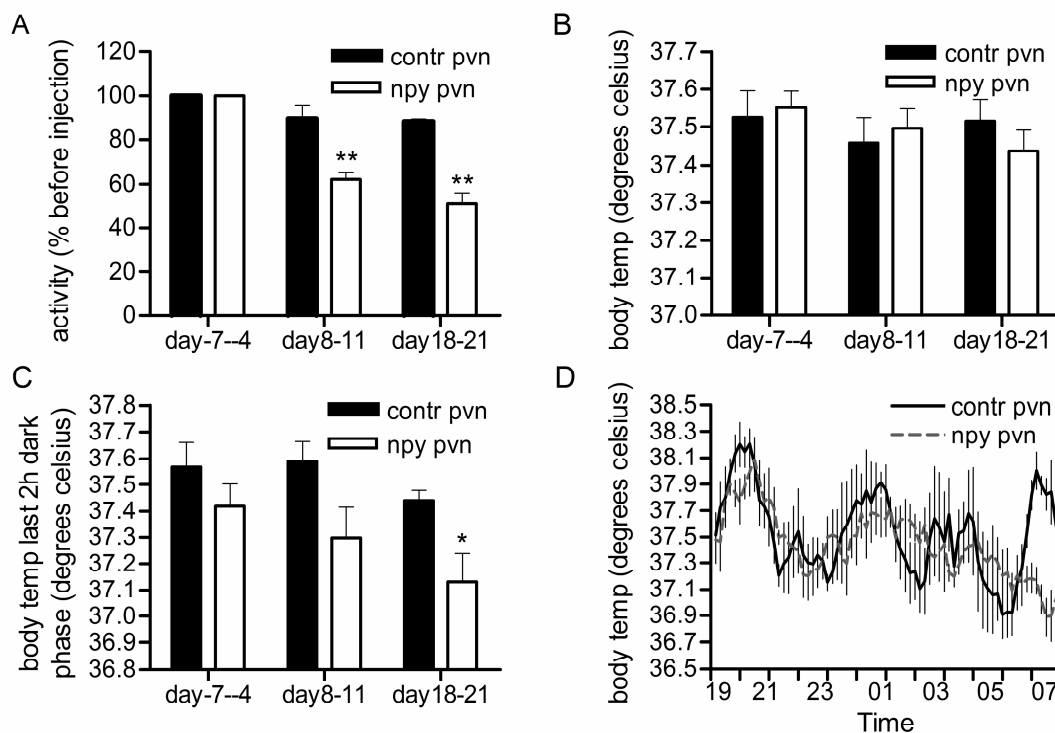**Figure 3:** Effects of rAAV-NPY injections on locomotor activity (A) and body temperature (B) in the total dark phase compared to rAAV-contr-PVN rats. C Effects of rAAV-NPY injections on body temperature in the last two hours of the dark phase. D. Example of temperature rhythm in the dark phase (day 18). Activity data are presented as percentage of values before injection. * p<0.05, ** p<0.01.

Effects of rAAV-NPY on body temperature and locomotor activity

Because an increase in FE suggests a reduction in energy expenditure, we examined body temperature and locomotor activity at the same days as FE was measured. Locomotor activity in the light period was similar in both groups (data not shown). However, during the dark period, rAAV-NPY-PVN rats showed a reduction of locomotor activity. One week after injection night-time activity of rAAV-NPY-PVN rats was only 62% of the activity individual rats displayed before injection, which was further reduced to $51 \pm 5\%$ at day 18-21 (versus $88 \pm 1\%$ for rAAV-contr-PVN rats, $p<0.01$) (Fig. 3A).

Besides a reduced locomotor activity in the dark period, rAAV-NPY-PVN rats tended to have a decline in body temperature in the dark phase. Although in the second week after injection there was no difference in body temperature of rAAV-NPY-PVN and rAAV-contr-PVN rats, at the end of the study there was a trend towards hypothermia in rAAV-NPY-PVN rats (Fig. 3B), which was significant at the last two hours of the dark phase ($p<0.05$) (Figs 3C and D). There was no significant effect of rAAV-NPY injection on body temperature in the light phase.

Effects of rAAV-NPY on endocrine parameters

Table 1 and Figure 4 summarize plasma levels of endocrine parameters measured at three time points after rAAV-NPY injections.

rAAV-NPY-PVN injections resulted in a significant elevation in basal concentrations of plasma leptin and insulin seven days after injection (Figs 4A and B). Plasma leptin concentrations in rAAV-NPY-PVN rats increased drastically over time, resulting in five times higher levels compared to control injected rats at the end of the study.

Plasma insulin concentrations were higher than controls in the first period after injection, resulting in three fold higher levels two weeks after injection. In the last week of the study, plasma insulin concentrations were still increased as compared to control groups. However, plasma insulin concentrations had also increased in the control groups as expected in sedentary rats fed ad libitum at this age.

Plasma adiponectin concentrations were not significantly changed seven days after rAAV-NPY injections. However, both two and three weeks after injections, rAAV-NPY-PVN rats showed increased circulating adiponectin concentrations (Fig. 4C).
rAAV-NPY-PVN injections did not produce any change in glycemia in the first two weeks after injection. However at day 23, these rats were slightly hyperglycemic when compared to rAAV-contr-PVN rats.

Plasma concentrations of FFA, TG, corticosterone and ACTH of rAAV-NPY-PVN rats were not significantly different from control values at any time point (Table 1).

Table 1: Effects of rAAV-NPY on endocrine parameters 7, 14 and 23 days after injections

	Day 7			Day 14			Day 23		
	contr-pvn	npy-thal	npy-pvn	contr-pvn	npy-thal	npy-pvn	contr-pvn	npy-thal	npy-pvn
gluc (mmol/l)	10.8±0.26	10.3±0.40	10.5±0.2	9.8±0.41	9.3±0.10	10.3±0.49	10.4±0.22	11.0±0.60	11.7±0.22$^{\#}$
FFA (mg/dl)	3.7±0.47	3.6±0.77	2.9±0.60	3.6±0.78	4.1±0.71	2.9±0.53	0.9±0.08	1.1±0.19	0.7±0.10
TG (mg/dl)	85.0±14.9	97.5±12.0	118.6±10.5	78.3±8.9	63.1±5.1	75.0±7.9	106.5±18.4	91.1±16.3	102.3±6.5
glyc (mg/dl)	32.5±5.1	33.2±9.8	26.5±6.9	37.1±13.2	30.2±5.6	20.3±3.8	8.6±1.4	6.3±1.2	7.3±0.9
cort (µg/dl)	10.6±2.53	4.0±1.57	12.8±3.03	10.7±5.86	6.9±4.44	17.8±5.35	18.3±2.44	14.6±3.20	13.8±3.00
ACTH (pg/ml)	48.2±4.76	42.0±2.34	41.8±2.05	27.8±3.10	37.5±21.0	49.6±10.1	39.6±2.96	53.3±12.7	36.9±8.11

Gluc, glucose; FFA, free fatty acids; TG, triglycerides; glyc, glycerol; cort, corticosterone; ACTH, adrenocorticotropic hormone. #$p<0.05$ vs contr-pvn
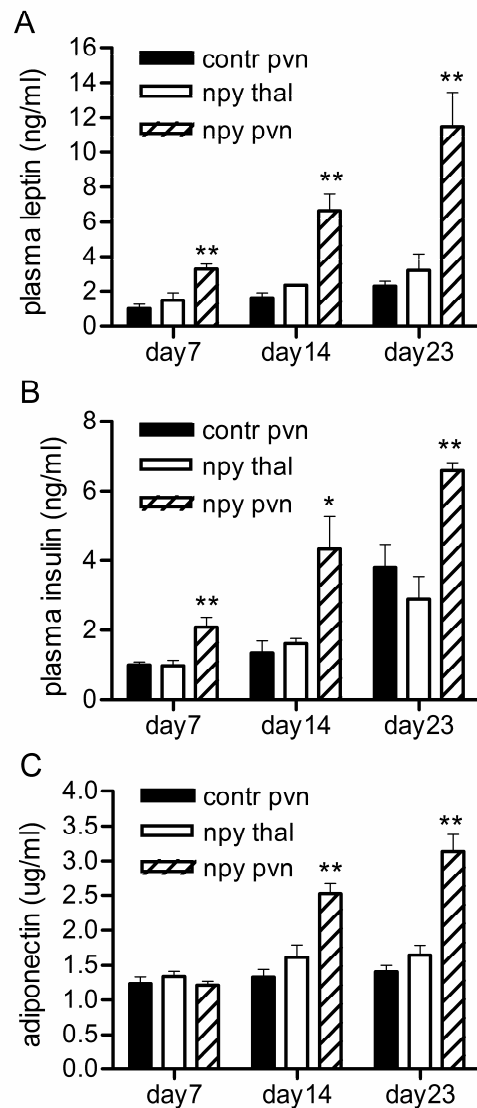
Figure 4: Effects of rAAV-NPY injections on basal plasma leptin (A), insulin (B) and adiponectin (Apn) (C) compared to rAAV-contr-PVN rats or rAAV-NPY-thal rats 7, 14 and 23 days after injection. * $p<0.05$, ** $p<0.01$.

Effects of rAAV-NPY on glucose tolerance

On day 17 after viral injections, we performed a glucose tolerance test. As shown in Figure 5A, glucose clearance after an intravenous glucose challenge was normal in rAAV-NPY-PVN rats. However, the insulin response to the glucose challenge was markedly increased (Fig. 5B), indicating a decreased insulin sensitivity.

Effects of rAAV-NPY on body composition

Table 2 and Figure 6 summarize body composition 23 days after viral injections. rAAV-NPY-PVN injections had a significant effect on the waist circumference of the rats, without influencing naso-anal length (Fig. 6A).

Total white adipose fat tissue (WAT) mass (retroperitoneal and epididymal) was significantly increased in rAAV-NPY-PVN rats as compared to both control groups (Fig. 6B). Additionally, interscapular BAT mass was also increased in rAAV-NPY-PVN rats (Table 2). Finally, rAAV-NPY-PVN rats had smaller adrenals as compared to control groups, but there was no significant change in thymus (Table 2).
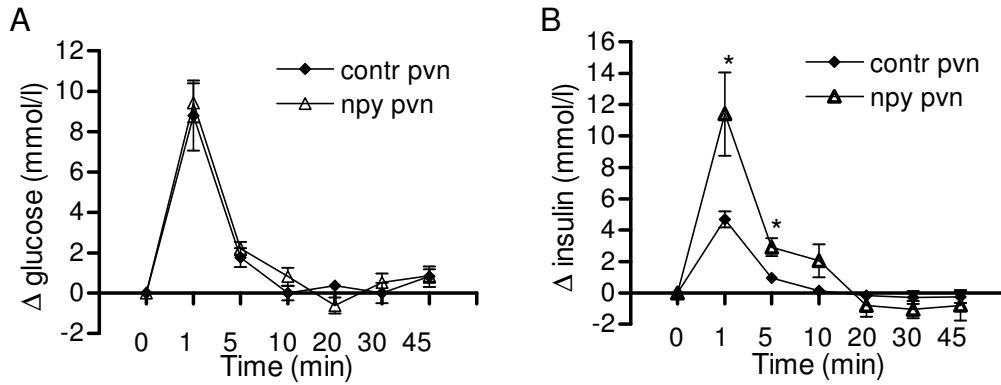
Figure 5: Effects of rAAV-NPY injections on blood glucose (A) and plasma insulin levels (B) compared to rAAV-contr-PVN rats following intravenous administration of glucose. * $p < 0.05$.
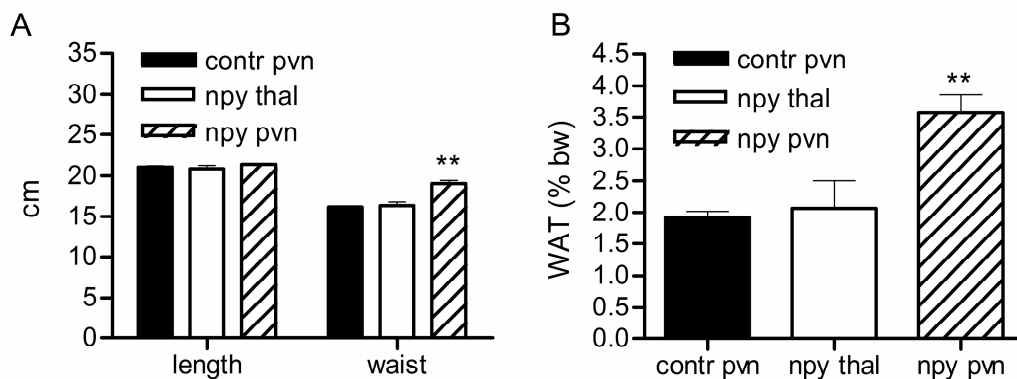
Figure 6: Effects of rAAV-NPY injections on naso-anal and waist size (A), retroperitoneal WAT depot (B), and liver weight (C) 23 days after injection compared to rAAV-contr-PVN rats or rAAV-NPY-thal rats. * $p < 0.05$, ** $p < 0.01$.

Table 2: Effects of rAAV-NPY on body composition 23 days after injections.

	Day 23		
	contr-pvn	npy-thal	npy-pvn
adrenals (‰bw)	0.14±0.01	0.15±0.01	0.11±0.01**
thymus (%bw)	0.10±0.022	0.13±0.011	0.12±0.010
BAT (%bw)	0.15±0.01	0.18±0.02	0.27±0.03**

** $p < 0.01$ vs. contr-pvn and npy-thal.

Effects of rAAV-NPY on neuropeptide expression in the arcuate nucleus

mRNA expression of NPY, AgRP and POMC in the arcuate nucleus three weeks after rAAV injections was measured by raISH. AgRP expression of rAAV-NPY-PVN rats was significantly reduced to 40% of control values, while POMC and NPY expression were unchanged (Fig. 7).
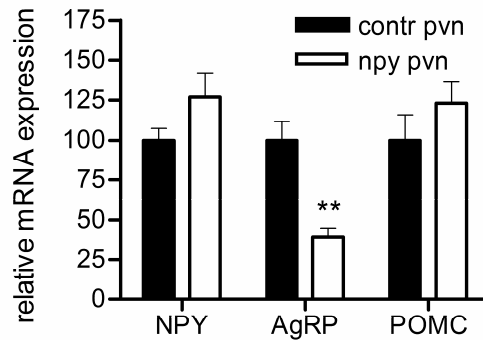
Figure 7: Effects of rAAV-NPY injections on arcuate nucleus expression of NPY, AgRP and POMC mRNA 23 days after injection compared to rAAV-contr-PVN rats. ** p<0.01.

DISCUSSION

In this study we show that rAAV-mediated overexpression in the PVN results not only in hyperphagia in the light phase, but also in changes in body temperature and locomotor activity in the dark phase. These changes together lead to an obese phenotype characterized by increased fat mass, elevated plasma concentrations of insulin, leptin and adiponectin, and decreased AgRP expression in the arcuate nucleus.

Interestingly, our data clearly show that the increased body weight gain is not solely due to increased food intake. In the first days when food intake was increased in rAAV-NPY-PVN rats, the rats showed a higher body weight gain than expected, compared to the amount of food eaten. This increased feed efficiency (FE) suggests that NPY overexpression in the PVN does not only stimulate food intake, but also reduces energy expenditure. Indeed, dark phase locomotor activity was reduced in rAAV-NPY-PVN rats, indicating that long-term elevated NPY signaling in the PVN is sufficient to reduce locomotor activity. This is a novel finding, since so far there are no reports of NPY administration into the PVN on locomotor activity in the home cage, although activity in the home cage or open field can be suppressed after ICV administration (209),. The fact that FE is no longer increased at the end of the experiment, in spite of a continuously lower activity, may be explained by the fact that rAAV-NPY-PVN rats had a significantly increased body mass, which requires more energy from food to maintain.

Another factor that can contribute to energy expenditure is thermogenesis. Several studies have clearly demonstrated that ICV NPY administration, or local NPY injections in

the PVN, decreases thermogenesis, by suppressing sympathetic activity to (208) and reducing UCPI mRNA in interscapular BAT (206;207). Although this suggests a NPY induced reduction in body temperature, results of NPY injections in the PVN are contradictory (205;223). Although we did not find effects of NPY on mean day time or night time body temperature, there was a significant reduction in body temperature in the late dark phase in rAAV-NPY-PVN rats. Our data show an increase in food intake restricted to the light period, whereas body temperature was only changed during the last 2h of the dark phase.

Others already reported that chronic icv NPY induces hyperphagia and obesity accompanied with high plasma concentrations of insulin and leptin (201;203;214). Interestingly, plasma concentrations of leptin and insulin rose before an effect of NPY on food intake and body weight could be observed. Already at day 7, rAAV-NPY-PVN rats showed elevated concentrations of plasma leptin and insulin, while food intake and body weight were still normal. Earlier acute ICV studies also showed this direct effect of NPY on insulin secretion (199-203), however, the direct involvement of the PVN was not shown before. In addition, the adipose tissue derived hormone adiponectin was not altered at this time point, suggesting a differential regulation of leptin and adiponectin.

At a later time point, rats injected with rAAV-NPY in the PVN showed insulin resistance; glucose clearance was normal after an intravenous glucose tolerance test, performed 17 days after virus injection, however, this was accompanied by an increased insulin response. Whether this is due to an increase in insulin secretion or a decrease in insulin clearance remains to be determined.

Although the effects of NPY on insulin secretion largely depend on food intake, with the largest effects occurring when food is available (199-201;203), NPY can also increase plasma insulin concentrations when eating is prevented (199;202;203). This food-independent increase in circulating insulin probably involves NPY-induced stimulation of the parasympathetic nervous system. Indeed, ICV NPY administration has been shown to activate the parasympathetic nervous system (346). Additionally, Buijs *et al.*, has injected pseudorabies virus in rats with a sympathetic denervation of the pancreas, and demonstrated the presence of vagal innervation from the PVN to the pancreas (347). Furthermore, it is known that the PVN is an important nucleus in the regulation of sympathetic nerve outflow (348). The PVN also innervates adipose tissue (349;350), and reduced sympathetic versus parasympathetic activity contributes to an increase in leptin concentrations (351-353). Therefore, it is plausible that the increase in leptin and insulin in the first period after rAAV-NPY-PVN injections results from NPY-induced vagal stimulation of the pancreas and WAT.

Despite the fact that others reported NPY-induced increases in HPA axis activity (203;354), long-term overexpression of NPY in the PVN did not alter plasma concentrations

of ACTH and corticosterone. The significant reductions in adrenal gland weight that we found even suggest decreased HPA axis activity. Although increased HPA axis activity has been associated with increased food intake and obesity (355), we did not find this to contribute to the obese phenotype described here.

rAAV-NPY-PVN rats showed a strong reduction in AgRP expression levels in the arcuate nucleus, presumably as a result of increased leptin signaling in this nucleus, since plasma levels of leptin were increased. The fact that the reduction in AgRP did not reduce food intake suggests that the elevated (viral mediated) NPY signaling in the PVN acts downstream of AgRP signaling.

Even though obesity is associated with low circulating adiponectin concentrations (352), there are no reports on the effects of central NPY administration on plasma adiponectin. Meanwhile, it has been shown recently that mice with increased adiponectin levels due to a mutation or transgenic overexpression indeed are obese (356;357). Since plasma adiponectin was increased in the rAAV-NPY-PVN rats at the end of the study, as well as WAT mass, it may be possible that the elevated adiponectin levels reflect increased WAT mass, which secretes adiponectin in the bloodstream.

We did not find a significant change in concentrations of FFA or TG at any time point of the study. This is consistent with previous studies which have shown that effects of chronically ICV infused NPY on fatty acids are transient (121;214).

While we show that long-term overexpression of NPY does have a phenotype, others reported that global overexpression of NPY from embryonic stage on did not result in changes in food intake and body weight gain in rats fed a normal chow (230;231;233). Compensatory changes during development or low NPY overexpression of NPY transgenic animals however may explain the lack of effect on body weight and food intake.

In summary, injection of rAAV-NPY in the PVN results in obesity, characterized not only by hyperphagia and increased fat storage, but also by elevated plasma leptin, insulin and adiponectin, decreased body temperature and hypoactivity. Although our earlier study with AAV-Agouti showed that rAAV-derived Agouti remained local (337), we cannot exclude the possibility that some of the viral-delivered NPY used in this study is transported to target sites of the PVN, explaining some of the described effects. The results suggest that NPY-induced obesity is not only the result of increased food intake, but also of increases in leptin and insulin that are partly food independent, and changes in locomotor activity and thermogenesis. This indicates that increased NPY expression in the PVN is sufficient to produce all parameters associated with obesity caused by increased NPY signaling in the brain, and strengthens the role of the PVN in regulation of energy balance by NPY.

ACKNOWLEDGEMENTS

Chapter 5

rAAV-mediated NPY overexpression in the mediodorsal hypothalamus reveals a role for NPY in body weight set-point in adult rats

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RAAV-MEDIATED NPY OVEREXPRESSION IN THE MEDIODORSAL HYPOTHALAMUS REVEALS A ROLE FOR NPY IN BODY WEIGHT SET-POINT IN ADULT RATS

ABSTRACT

In contrast to strong pharmacological effects of NPY on body weight gain, germline overexpression or deletion of the NPY gene has only modest effects. Using another experimental approach, we bypassed developmental compensations that may be induced with germline NPY overexpression, and injected rAAV-NPY in the mediodorsal hypothalamus. We examined body weight gain and food intake for 50 days in rats that were either pair-fed or fed *ad libitum*.

Rats injected with rAAV-NPY increased food intake for four weeks, where after it normalized again, but had a continuous reduction in activity and body temperature, independent from food intake. We only found a compensatory effect on AgRP mRNA levels, whereas POMC, TRH, CRF, Orexin, MCH and endogenous NPY (in the arcuate nucleus) were unchanged. As soon as a certain body weight was reached following increased NPY signaling, compensatory mechanisms were triggered and food intake was normalized.

Rats that were pair-fed for three weeks and then allowed *ad libitum* food, increased their food intake, which normalized when the rats had reached the same body weight as rats that were *ad libitum* fed the entire study. These data support a role for NPY in body weight set-point regulation.

INTRODUCTION

Neuropeptide Y (NPY) is one of the most abundant neuropeptides in the brain. NPY-ergic neurons that are involved in in the regulation of energy balance are mainly expressed in the arcuate nucleus and project to a variety of areas, including the paraventricular (PVN), lateral (LH) and dorsomedial (DMH) nuclei of the hypothalamus and the medial preoptic area (56;177-179). Chronic central administration of NPY results in an obese phenotype, characterized by hyperphagia, increased lipogenesis in liver and adipose tissue and elevated plasma concentrations of leptin, insulin and corticosterone (121;201;203;212;214). Consistently, suppression of endogenous NPY levels by antisense oligonucleotides reduces body weight gain, food intake and insulin secretion (215;218;219;358).

Unexpectedly, NPY transgenic animal models show normal food intake and body weight gain when fed on regular chow (230;231;233). Whether this is due to a developmental change, compensation by counter-regulatory mechanisms or only limited overexpression is not known. Interestingly, also in models where the genes for NPY or one of its receptors are disrupted there are no, or even unexpected phenotypes with regard to body weight and food intake. Deletion of NPY has no effect on food intake and body weight gain, unless animals are fasted for 24 or 48 hours (235;238), suggesting that NPY is more important for situations when food is scarce than for normal feeding behavior. Models where one of the NPY receptors is knocked out (Y1$^{-/-}$, Y2$^{-/-}$ and Y5$^{-/-}$) surprisingly show no effect on or even an increase in body weight and feeding behavior (185;191;246). This suggests that a chronic

absence or overexpression of NPY during development results in compensation by other systems and indicates a remarkable plasticity of the systems involved in energy balance during development. Indeed, it has been shown recently that ablation of arcuate NPY/AgRP neurons decreases food intake in adult mice, but that they can be destroyed without any effect in neonates (155;156). Taken together, results obtained after pharmacological and genetic studies on the function of NPY are contradictory. Compensatory pathways may only be activated after a longer period of changes in the levels of NPY. This is however difficult to test pharmacologically, because it is not feasible to reliably infuse ligands in local brain regions for more than a week.

Recently, it was shown that if NPY is overexpressed at the adult stage in the hypothalamus of mice, obesity is induced (253). These results are comparable to the results after chronic infusion of NPY, and show that if NPY is overexpressed after development, obesity is induced. Apparently, compensatory mechanisms counteract increased NPY signaling during development, but have limited capacity in adulthood.

In order to investigate whether compensation can also take place in the adult rat after a longer period of NPY overexpression, we injected rAAV-NPY in the mediodorsal hypothalamus and examined body weight gain and food intake for 50 days. By carefully monitoring feeding behavior, locomotor activity and body temperature under *ad libitum* and pair-fed conditions we aimed to unravel how NPY induces obesity and to what extent increased food intake contributes to the obese phenotype.

To identify whether the effects of NPY overexpression on body weight and food intake are compensated for, we measured plasma levels of leptin and insulin, and analyzed gene expression levels of neuropeptides in the arcuate nucleus.

MATERIAL AND METHODS

Animals

Male Wistar rats weighing 220-250 g were purchased from Charles River (Crl-Wu, Germany). They were individually housed in filtertop cages with *ad libitum* access to food and water. Animals were kept in a temperature and humidity controlled room (21±2 °C) under a 12h/12h light/dark cycle (lights on at 0700 h). All experimental procedures were approved by the Committee for Animal Experimentation of the University of Utrecht, Utrecht, The Netherlands.

Experiment 1

Rats were anesthetized with 0.1 ml/100 g im hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and 0.05 ml/100 g ip dormicum (Hoffman-LaRoche, Mijdrecht, The Netherlands). Transmitters (TA10TA-F40 Data Science International, St Paul, Minnesota, USA) were placed in the abdominal cavity. Rats were left to recover for three weeks.

Seven days after baseline recordings, rats were anesthetized again as described above. Using a stereotax, rAAV-NSE-NPY (AAV-NPY) (n=9) or rAAV-NSE-empty (AAV-contr) (n=6) was injected bilaterally into the mediodorsal hypothalamus, with coordinates aiming for the PVN (AP: -1.8 mm from bregma, ML: ±0.3 mm from bregma, and DV: -8.0 mm below the skull). The virus was a kind gift of M.J. During (New York). Production of AAV-NPY has been described previously (339;340). 1 µl of virus (1×10^8 genomic copies) was injected per site over five minutes, after which the needle was kept in place for ten minutes before removal. After each surgery, rats received an injection with 5 mg/kg carprofen (Vericore Ltd, Dundee, United Kingdom). In this experiment, all rats were provided with *ad libitum* chow.

Experiment 2

In a second experiment, the first experiment was repeated, however, a part of the animals injected with AAV-NPY were pair-fed to the control animals. Pair feeding consisted in providing the rats every day the same amount of chow that was eaten by the control animals. Therefore, the pair-fed animals were injected with the virus two days later than the control group.

In total, 18 animals were injected with AAV-NPY, and 6 animals with AAV-contr in the PVN. Animals injected with AAV-contr and 9 animals injected with AAV-NPY were fed *ad libitum* during the experiment (AAV-contr and AAV-NPY-al). The other 9 animals injected with AAV-NPY were pair-fed to the control group (AAV-NPY-pf). Animals in experiment 2 had a slightly increased body weight at the moment of injection than animals used in experiment 1.

Data analysis

One week before, until fifty days after viral injections, body weight, food intake, body temperature and locomotor activity were recorded.

Body weight gain and food intake were measured daily at 11.00h. Locomotor activity and body temperature were measured via the transmitters that send digitized data via radio frequency signals to a nearby receiver. The data were automatically recorded every ten minutes, and averaged per hour using DSI software (DSI, St Paul, MN).

Collection of blood and tissues

At day 50 after infection, rats were decapitated, trunk blood was collected in heparinized tubes after adding 83 µmol EDTA and 1 mg aprotinin, and immediately placed on ice. Plasma samples were stored at -20 °C until further analysis.

Brains were immediately removed after decapitation, quickly frozen in cold isopentane (-35 °C) and stored at -80 °C. Retroperitoneal and epididymal white adipose tissue (WAT) was isolated and weighed.

Verification of injection sites

16 μm coronal sections of the hypothalamus were sliced using a cryostat (Leica, Rijswijk, The Netherlands), thaw-mounted onto RNAse free Superfrost slides (Menzel, Germany) and stored at -80 °C. Viral infection was localized by in situ hybridization (ISH) with a digoxigenin (DIG)-labeled woodchuck post-transcriptional regulatory element (WPRE) probe. The WPRE sequence is part of the expression cassette of all vectors used here. ISH procedure has previously been described (341). Results from rats with incorrect injections were excluded from the analysis.

Quantitative in situ hybridization

16 μm coronal sections were used for raISH. 33P-labeled antisense RNA probes were made using AgRP (396 bp mouse (343)), NPY (286 bp rat (344)), POMC (350 bp rat (343)), CRF (770 bp rat), TRH, MCH (500 bp mouse (359)) and prepro-orexin (542 bp rat, AF041241) cDNA fragments. raISH procedure has previously been described (345). Expression of AgRP, NPY and POMC was analyzed in the arcuate nucleus, expression of CRF and TRH was analyzed in the PVN and expression of MCH and prepro-orexin was analyzed in the LH using Image J software (National Institutes of Health, Bethesda, Maryland, USA). Expression of NPY was also measured in the mediodorsal hypothalamus to confirm viral-mediated overexpression.

Plasma analysis

Plasma leptin and insulin were analyzed in duplicate using radioimmunoassay kits, (Linco Research, St Charles Missouri, USA).

Statistical analysis

Data are presented as group means ± SEM. Differences in body weight, food intake, body temperature and locomotor activity were assessed using repeated measure analysis. When significant overall interactions were found, post hoc analyses were performed with T-tests or one-way ANOVA. Differences between fat weight, leptin, insulin and gene expression levels were analyzed by T-tests or one-way ANOVA, followed by Tukey's post-hoc test. Differences were being considered significant at $p < 0.05$.

RESULTS

Localization of rAAV in the brain

In order to overexpress NPY in the mediodorsal hypothalamus, we bilaterally injected AAV-NPY or AAV-contr with coordinates aimed for the PVN in the hypothalamus of rats. Correct injection was verified by WPRE mRNA expression (Figs 1A and B), and animals with incorrect injections were excluded from analysis (in total, in experiment 1 three rats from AAV-NPY were excluded, and in experiment 2 one rat from AAV-contr, two rats from AAV-NPY-al, and three rats from AAV-NPY-pf were excluded). Viral-induced NPY expression in the mediodorsal hypothalamus at fifty days post injection was confirmed by RA-ISH (Figs 1C and D). Viral-induced NPY mRNA expression in the mediodorsal hypothalamus of AAV-NPY injected rats was 167.6 ± 12.42 % of arcuate nucleus expression of controls (data not shown).
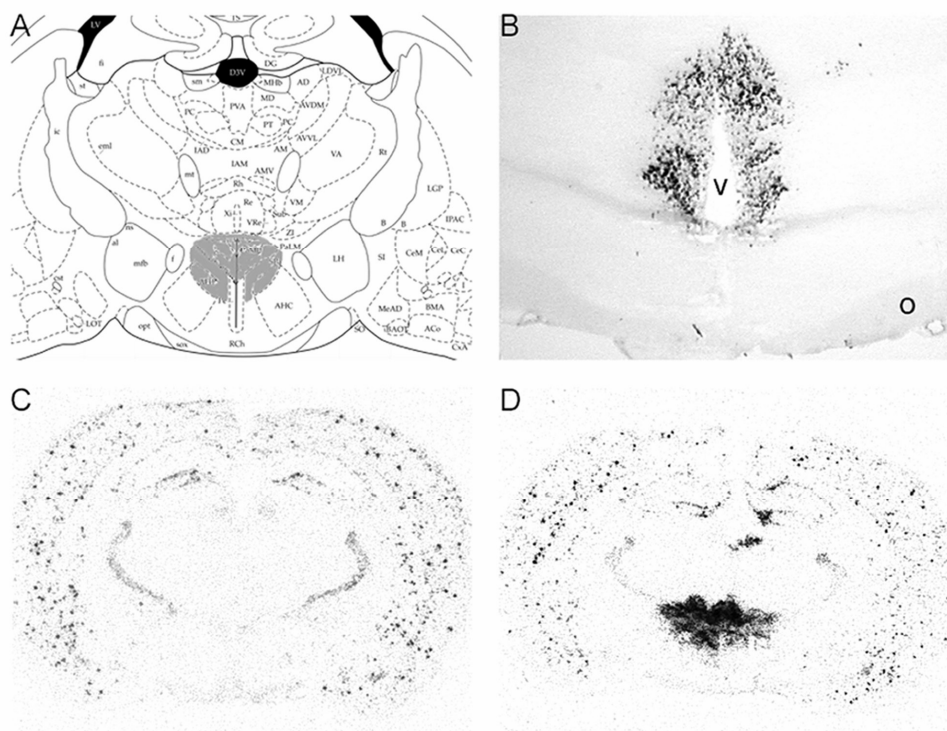
Figure 1: A. Schematic example of the injection area (indicated in grey) of the viral particles; B. Example of WPRE expression in the mediodorsal hypothalamus, as assessed by *in situ* hybridization (o = optic tract, v = third ventricle), adapted from Paxinos and Watson (321); C. Example of NPY mRNA expression in the mediodorsal hypothalamus of a rat injected with AAV-contr; D. Example of NPY mRNA expression in the mediodorsal hypothalamus of a rat injected with AAV-NPY.

Overexpression of NPY in the mediodorsal hypothalamus temporary increases food intake and body weight

Following injection of the rAAV particles in the mediodorsal hypothalamus, all animals showed a similar drop in body weight and food intake. Body weight and food

intake were back to pre-surgery levels within one week after injection. Food intake was increased in rats injected with AAV-NPY from day 7. Daily food intake increased until day 15, when it was 1.6 times higher than control animals. Food intake remained stable between day 15 and day 27, after which it slowly decreased until it was comparable to AAV-contr injected animals from day 40 post injection until the end of the study (Fig. 2A).

Figure 2: Effects of rAAV-NPY injection in the mediodorsal hypothalamus on daily food intake (A), cumulative body weight gain (B) and average body weight gain/day; (C). * p<0.05, ** p<0.01.

From day 9 after injection, rats injected with AAV-NPY showed an increase in body weight gain, which resulted in an accumulated body weight gain of 178% more than controls at the end of t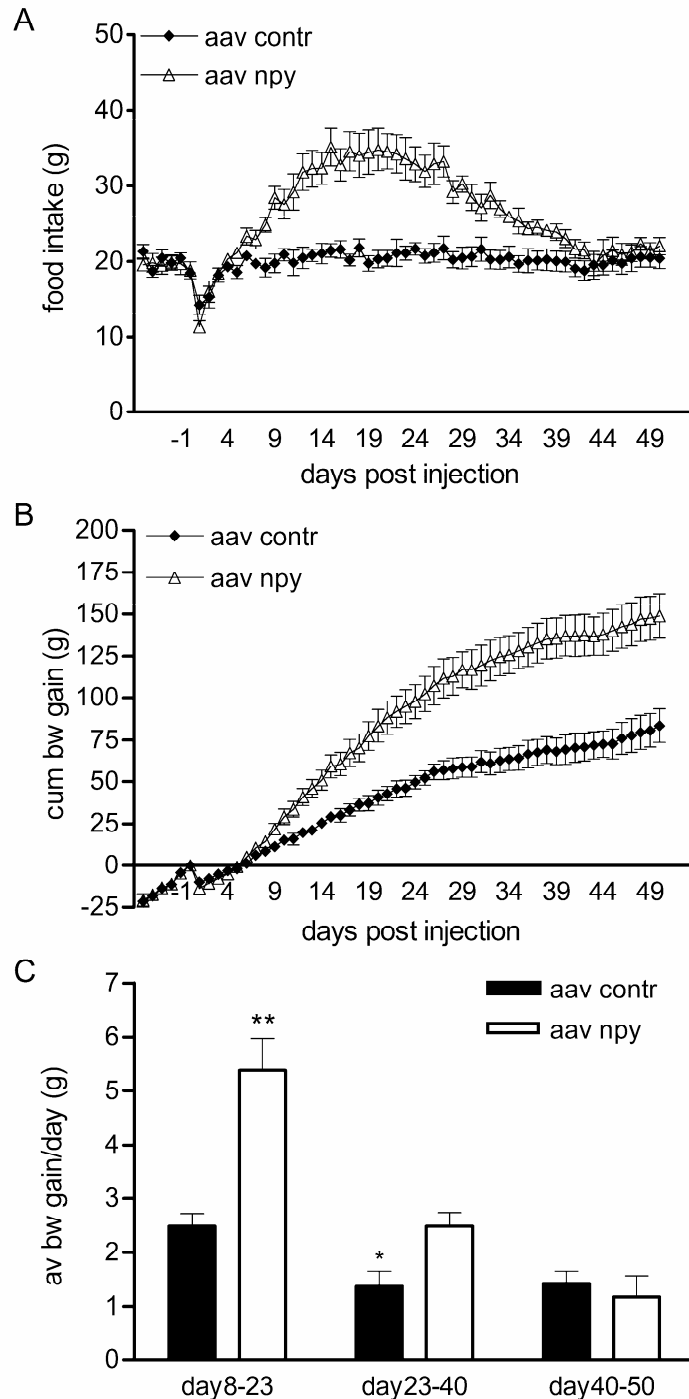he study (Fig. 2B). Differences in body weight gain of rats injected with AAV-NPY and AAV-contr were highest between day 8 and day 23 (average of 5.38 ± 0.60 vs 2.49 ± 0.24 g/day for AAV-NPY vs AAV-contr, p<0.01), slowly decreased between day 23 and 40 (average of 2.49 ± 0.26 vs 1.36 ± 0.29 g/day for AAV-NPY vs AAV-contr, p<0.05) and was not significant anymore in the last ten days of the study (average of 1.17 ± 0.39 vs 1.42 ± 0.23 g/day for AAV-NPY vs AAV-contr) (Fig. 2C).

Table 1: Effects of overexpression of NPY in the mediodorsal hypothalamus on body weight gain, relative WAT, and plasma concentrations of leptin and insulin, 50 days post injection.

	Bw gain (g)	WAT (%bw)	Leptin (ng/ml)	Insulin (ng/ml)
aav-contr	83.5 ± 9.99	3.17 ± 0.35	4.76 ± 0.67	3.10 ± 0.67
aav-npy	148.9 ± 13.06**	5.64 ± 0.39**	20.63 ± 4.61**	5.84 ± 0.75[#]

[#] p=0.08 vs contr, ** p<0.01 vs contr

Compensatory effects of NPY overexpression

Since there is still an obvious overexpression of NPY in the mediodorsal hypothalamus at day 50, which is comparable to overexpression at day 23 (145.0 ±10.1% of arcuate nucleus expression of controls, B. Tiesjema, unpublished observations), the reduction of effects on food intake and weight gain are not due to decreased viral-mediated overexpression of NPY.

The increased body weight gain and WAT stores were accompanied by increased plasma leptin and insulin levels (table 1). Leptin levels were significantly elevated in rats injected with AAV-NPY (20.63 ± 4.61 vs 4.76 ± 0.67 ng/ml for AAV-NPY vs AAV-contr, p<0.01). There was also a tendency towards hyperinsulinemia in rats injected with AAV-NPY (5.84 ± 0.75 vs 3.10 ± 0.67 ng/ml for AAV-NPY vs AAV-contr, p=0.08).

Next we measured expression of well known orexigenic and anorexigenic neuropeptides in the hypothalamus in order to determine to what extent these systems adapt to NPY overexpression and increased leptin signaling. We have shown earlier that 23 days after AAV-NPY injections in the PVN, AgRP mRNA expression is reduced to 40% when compared to controls, while NPY and POMC expression is similar to controls (Chapter 4). The mRNA expression of NPY, AgRP and POMC in the arcuate nucleus was measured with raISH 50 days after injection of the viral particles. Although there was no significant difference in mRNA expression of NPY or POMC in the arcuate nucleus of rats injected with AAV-NPY when compared to control animals, AgRP expression was significantly reduced to 50% of control values (Fig. 3a). Neuropeptide expression in the PVN (CRF and TRH) and in the lateral hypothalamus (MCH and orexin) were not altered in animals injected with AAV-NPY (Figs 3B and C).
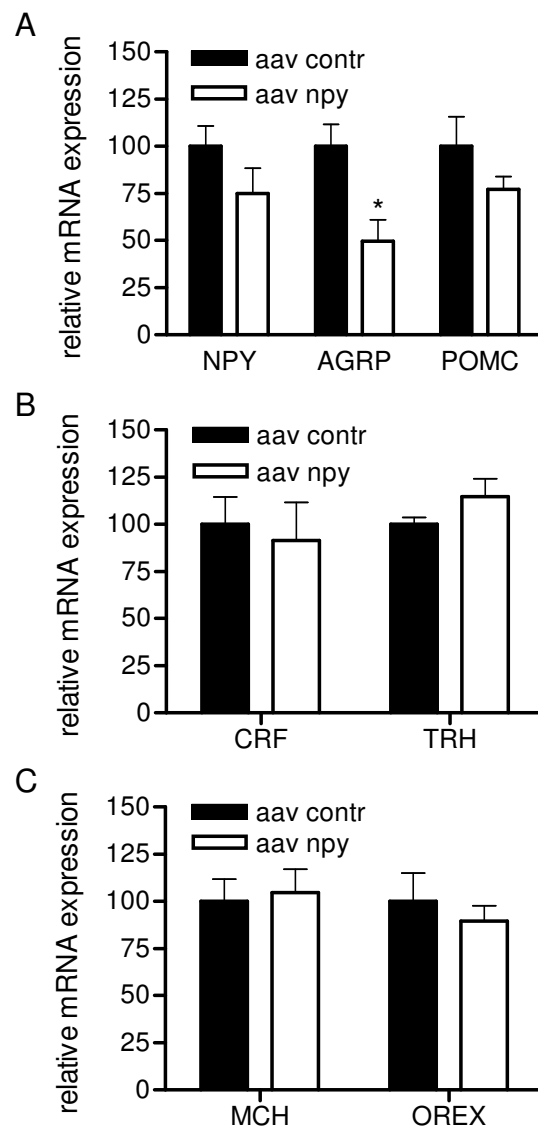
Figure 3: Effects of rAAV-NPY injection in the mediodorsal hypothalamus on mRNA expression of NPY, AgRP and POMC in the arcuate nucleus (A), CRF and TRH expression in the PVN (B) and MCH and orexin expression in the LH (C). * $p<0.05$.

Food independent decrease of body temperature and locomotor activity by NPY overexpression in the mediodorsal hypoyhalamus

To determine to what extent the effects of NPY overexpression on body weight gain and energy expenditure were dependent on the increase in food intake, a pair-fed study was performed (experiment 2). As shown in figure 2, also a new group of rats that were injected with AAV-NPY and fed *ad libitum* increased their food intake until day 18, whereafter it was stable for about 10 days before it normalized (Fig. 4A). Body weight gain increased until day

27, thereafter it was not significantly different anymore from controls (Figs 4B and C). This is consistent with the results from experiment 1.

Figure 4: Effects of rAAV-NPY injection in the mediodorsal hypothalamus on daily food intake (A), cumulative body weight gain (B) and average body weight gain/day (C) in rats that are either pair-fed or fed *ad libitum*. [#] p=0.07, * p<0.05, ** p<0.01.

AAV-NPY rats that were pair-fed to the AAV-contr rats, showed a decreased body weight gain when compared to controls (Figs 4B and C), which resulted in a cumulative

body weight gain that was significantly lower than AAV-contr rats twenty days after injection (12.28 ± 5.22 vs 33.9 ± 2.12 g for AAV-NPY-pf vs AAV-contr rats, p<0.01). The rats that were pair-fed consumed all their food in the first half of the dark period (data not shown).

Effects of AAV-NPY on body temperature are shown in Figures 5A and B. Although light phase body temperature tended to be increased in AAV-NPY injected animals, this was not significant (Fig. 5A). However, in the dark phase, body temperature was significantly reduced in AAV-NPY injected animals, starting two weeks after injection (Fig. 5B). Body temperature was equally decreased in *ad libitum* fed and pair-fed animals (Note that the AAV-NPY-pf group is pair-fed until day 21, where after it is fed *ad libitum*). Dark phase body temperature remained significantly reduced during the entire experiment.

Light phase locomotor activity of rats injected with AAV-NPY was not significantly different from animals injected with AAV-contr (Fig. 5C). Dark phase activity was reduced in both AAV-NPY-al and AAV-NPY-pf animals on day 6, and remained significantly reduced throughout the rest of the experiment (Fig. 5D).
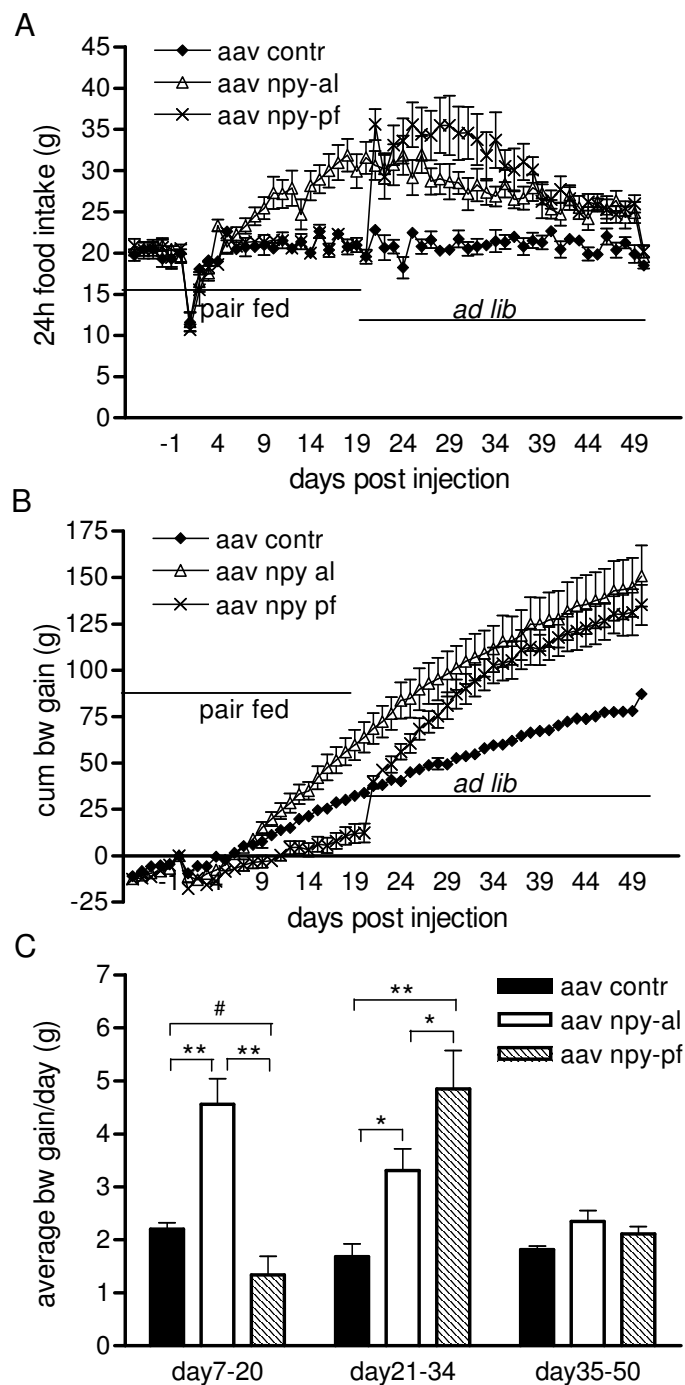

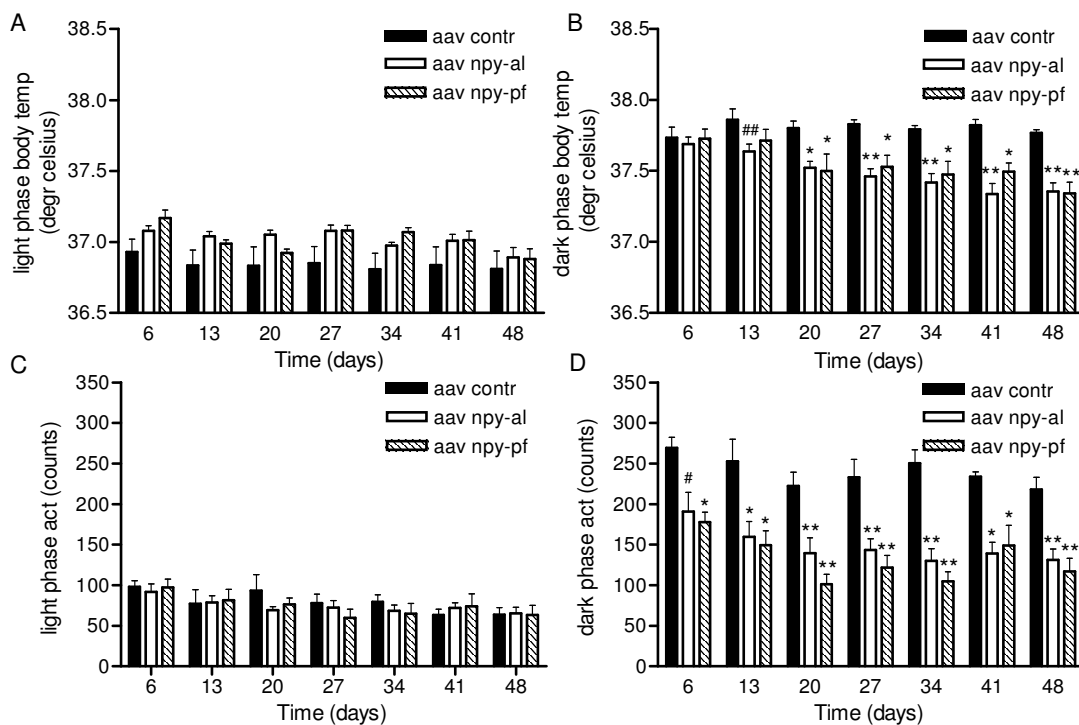**Figure 5:** Effects of rAAV-NPY injection in the mediodorsal hypothalamus on body temperature in the light (A) and dark phase (B), and locomotor activity in the light (C) and dark phase (D). #p=0.06, ## p=0.07, * p<0.05, ** p<0.01.

Involvement of downstream pathways

To determine whether the normalization of food intake observed in ad libitum fed AAV-NPY injected rats was caused by adaptations downstream of viral-induced NPY

signaling, we allowed the pair-fed animals to eat *ad libitum* from day 21 until the end of the experiment. We reasoned that, if adaptations downstream of NPY were responsible for the normalization of food intake, animals that were pair-fed for the first three weeks of the experiment would follow the food intake curve of the AAV-NPY-al rats from the moment they were given access to *ad libitum* food. Therefore, they should show hyperphagia (to a similar amount of AAV-NPY-al rats) for only one week, and normalize their food intake at the same time of AAV-NPY-al rats, irrespective of their body weight.

Food intake of AAV-NPY-pf animals immediately increased when they were fed *ad libitum*, and food intake remained elevated and stable until day 34 after injection, after which food intake returned to control values (Fig. 4A). Cumulative food intake from day 21-34 of AAV-NPY-pf rats was increased when compared to AAV-NPY-al rats (475.2 ± 39.0 g vs 388.5 ± 16.5 g, $p<0.05$).

In line with this, directly after being fed *ad libitum*, AAV-NPY-pf rats started to increase their body weight gain. Already twenty-four hours after their first day with *ad libitum* food, cumulative body weight gain of AAV-NPY-pf rats was similar to that of control rats (day 21: 39.6 ± 3.38 vs 37.7 ± 1.77 g for AAV-NPY-pf vs AAV-contr rats), and as of 4 days after *ad libitum* food their cumulative body weight gain was significantly higher than controls (day 24: 60.6 ± 5.12 vs 45 ± 2.33 g for AAV-NPY-pf vs AAV-contr rats, $p<0.05$). Between day 21 and 34 body weight gain per day was also increased when compared to AAV-NPY-al animals ($p<0.05$) (Fig. 4C). From day 34 post injection, body weight gain of the AAV-NPY-pf rats slowed down again, and average body weight gain per day was not different anymore from controls and AAV-NPY-al rats.

Fifty days after injection of the viral particles, body weight gain and fat percentage of AAV-NPY-pf animals was similar to that of AAV-NPY-al animals, and significantly increased when compared to control animals (Table 2). Furthermore, plasma concentrations of leptin and insulin were also elevated in comparison with control animals, but not different from AAV-NPY-al animals (Table 2).

Table 2: Effects of overexpression of NPY in the mediodorsal hypothalamus on body weight gain, relative WAT, and plasma concentrations of leptin and insulin in pair-fed and *ad libitum* fed rats, 50 days post injection.

	Bw gain (g)	WAT (%bw)	Leptin (ng/ml)	Insulin (ng/ml)
aav-contr	87.2 ± 1.38	2.18 ± 0.21	4.44 ± 0.85	4.44 ± 0.66
aav-npy-al	$150.8 \pm 16.45^*$	$5.40 \pm 0.53^{**}$	$18.58 \pm 3.86^*$	$7.86 \pm 0.51^{**}$
aav-npy-pf	$135.3 \pm 10.91^*$	$5.18 \pm 0.57^{**}$	$17.81 \pm 2.62^*$	$7.34 \pm 0.89^*$

* $p<0.05$ vs contr, ** $p<0.01$ vs contr

DISCUSSION

The results described in this study show that long-term overexpression of NPY in the mediodorsal hypothalamus results in a transient increase in food intake and body weight

gain, and a continuous reduction in dark phase locomotor activity and body temperature. When permitted to eat *ad libitum*, food-restricted AAV-NPY injected animals adjust their weight and fat mass to a similar level as non-restricted rats. This implies that increased NPY signaling in the mediodorsal hypothalamus alters body weight set-point. Furthermore, the decline in body temperature and locomotor activity was also observed in pair-fed rats, which indicates that the effects of NPY on energy expenditure are independent from food intake.

Approximately four weeks after virus injection, food intake of AAV-NPY injected rats that were fed *ad libitum* decreased, until it was comparable to intake of control rats. At the same time, body weight gain returned to normal values. This reduction of effects on food intake and weight gain was not due to a decreased amount of viral-derived NPY expression, since NPY mRNA levels in the mediodorsal hypothalamus on day 23 and day 50 were similar. Furthermore, effects on dark phase body temperature and locomotor activity were continuous, also indicating that the extent of viral-mediated NPY overexpression at the end of the experiment was still high enough to elicit effects.

The normalization of food intake and body weight gain in the last phase of our study suggests that counter-regulatory mechanisms are triggered when NPY is overexpressed during the adult stage. Other studies suggest that adaptations to changes in the NPY system can only occur during development or in early stages of life, and that the plasticity of the systems involved in energy balance declines with age (155;156). Nevertheless, the normalization of food intake and body weight gain weeks after the injection of AAV-NPY, as observed in our study, suggests that compensatory pathways are also activated in adult animals, however, it takes some time before adaptation begins. This explains why compensatory effects are not observed after 1 week administration of NPY, which is simply too short to initiate the counter-regulatory mechanisms. Although it is clear that adaptations occur after a chronic situation of imbalance in the NPY system, the mechanisms behind these adaptations are poorly understood.

The adiposity signals leptin and insulin are well known regulators of appetite. When body fat mass increases, plasma levels of leptin and insulin rise (8;9). This results in stimulation of POMC neuronal activity and inhibition of NPY/AgRP neurons in the arcuate nucleus, and thus a decrease in food intake (17). A logical explanation for the normalization of food intake as described above would therefore be that, due to the increased fat mass, plasma concentrations of leptin and insulin rise in AAV-NPY injected rats. Subsequently, this would alter the gene expression patterns of neuropeptides in the arcuate nucleus. Indeed, fat mass and plasma levels of leptin and insulin of AAV-NPY injected rats are elevated at day 50, and although POMC and NPY mRNA expression in the arcuate nucleus was not significantly altered, AgRP expression was reduced. Since AgRP mRNA levels were similarly reduced at 23 and at 50 days of NPY overexpression, compensation via reduced

levels of AgRP mRNA can not explain why food intake was lower at 50 days. However, we cannot exclude that the increased levels of leptin affect other neural circuits that are responsible for the normalization of food intake.

An alternative explanation for the normalized food intake at the end of the experiment is that the increased NPY signaling alters body weight setpoint. The theory that a peptide can adjust the degree of food intake to the current level of body adiposity was first described by Powley and Keesey (360). They hypothesized that an adiposity signal only alters food intake for the period that is needed to achieve the adiposity status that is characteristic for the specific signal, regardless of the initial body weight of the animal (360). Examples of such signals are insulin and MTII (a melanocortin agonist), which only reduce food intake or body weight gain to the extent that is needed to reach a new defended degree of adiposity (361;362). Therefore, these ligands are more effective in animals with a normal weight than in animals that are weight reduced by food restriction (361;362). Since AAV-NPY injected animals that were pair-fed to controls for three weeks before they were allowed *ad libitum* food, increased their food intake until they reached the same body weight as AAV-NPY-al rats, we favor the hypothesis that increased NPY expression increased their set-point to a certain amount of body weight or adiposity. When this set-point is achieved, sensitivity for NPY decreases and food intake normalizes. Since the defence of a certain body weight set-point is also observed in humans (363), it is possible that in some subjects obesity is maintained despite a normal pattern of food intake, by a chronically altered NPY signaling.

Even though food intake of AAV-NPY injected rats normalizes over time, body temperature and locomotor activity remain decreased, indicating that not all effects caused by the high levels of NPY in the hypothalamus are compensated for. Although data on locomotor activity or body temperature in NPY transgenic animals are not reported, NPY^{-/-} animals show no change in these parameters (237;244), suggesting that, if alterations in temperature and activity occur due to absence of NPY during development, they are masked by the contribution of counter-regulatory systems. Since animals with an altered NPY signaling during development have a normal body weight, it appears that altered NPY signaling in prenatal animals does not affect body weight set-point in a similar manner as in adults. Therefore, it is likely that compensatory mechanisms during development differ from the mechanisms in the adult rat. However, it is possible that the secondary mechanisms responsible for the adaptation to either too high or absent NPY levels in the brain are similar.

Although it is reported that one week of ICV administration of NPY does not alter body weight gain when increased food intake is prevented (121;203), AAV-NPY injected rats that were pair-fed to controls showed a lower body weight gain when compared to AAV-contr rats. AAV-NPY-pf rats ingested all their food in the first half of the dark period. Since

NPY overexpression specifically stimulates light phase food intake (Chapter 4), the absence of food in the light phase might be perceived as starvation. Although dark phase central body temperature was decreased in the pair-fed rats, as assessed using telemetry probes, it is possible that the stressful condition of the pair-fed rats increases heat loss via the extremities, which would not be detected by the telemetry probes. In addition, we can not exclude that in pair-fed rats calories are not as efficiently taken up as in control animals, since stress inhibits gastric motility and increases colonic motor function and defecation (364).

In summary, this study provides evidence that NPY overexpression in the mediodorsal hypothalamus of adult rats plays a role in regulation of body weight setpoint. This causes a transient increase in food intake and body weight gain, but an unremitting (food independent) reduction of energy expenditure of rats injected with AAV-NPY. The results support the data that, also in adult rats, the systems involved in the regulation of energy balance show enough plasticity to compensate for the effects of high NPY signaling on feeding behavior.

Chapter 6

Differential effects of rAAV-mediated NPY overexpression in the PVN and LH on energy balance

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DIFFERENTIAL EFFECTS OF RAAV-MEDIATED NPY OVEREXPRESSION IN THE PVN AND LH ON ENERGY BALANCE

ABSTRACT

The PVN and LH/PFA are in varying degrees involved in the acute, hyperphagic effects of NPY. Nevertheless, implication in energy expenditure and long-term effects of NPY are less understood. In order to further clarify the role of the PVN and the LH/PFA in NPY-induced obesity, we injected AAV-NPY in the PVN or LH/PFA of adult rats. Animals were then followed for 50 days.

Although injections in both areas resulted in obesity, caused by increased food intake and decreased energy expenditure, clear differences were observed in the manner via which obesity develops. Whereas food intake and body weight gain of PVN-NPY rats was only temporarily increased, LH-NPY rats remained hyperphagic for the entire 50 days. Additionally, AAV-NPY injections in the PVN only increase meal frequency, while LH/PFA injections alter both frequency and size. Moreover, in LH-NPY rats, but not PVN-NPY rats, circadian rhythmicity with regard to food intake and body temperature was lost. These data suggest that the NPY system differentially regulates energy intake and energy expenditure in the PVN and LH/PFA, which together adjust energy balance.

INTRODUCTION

Neuropeptide Y (NPY) is widely expressed in the brain and is involved in several aspects of the regulation of energy homeostasis. Besides having a strong, positive effect on food intake, NPY is also able to reduce metabolic rate and thermogenesis (193;206;208;220;335). Chronic intracerebroventricular (ICV) infusion of NPY results in obesity, characterized by hyperphagia, increased lipogenesis in liver and adipose tissue and elevated plasma concentrations of leptin, insulin and corticosterone (121;201;203;212;214).

NPY neurons involved in energy balance are mainly found in the arcuate nucleus (Arc), and project to a variety of areas, including the paraventricular nucleus of the hypothalamus (PVN) and the lateral (LH) / perifornical area (PFA). Also the dorsomedial nucleus of the hypothalamus (DMH) and medial preoptic area receive NPY-ergic terminals from the Arc (56;177;178). Most studies on the local role of NPY have focused on the PVN, due to the high amount of NPY-ergic terminals that are present in that nucleus (178). Nevertheless, the strongest feeding response is observed after injection of NPY into another area, i.e. the PFA.

Although these nuclei are in varying degrees involved in the acute hyperphagic effects of NPY, implication in energy expenditure and long-term effects of NPY are less understood. The acute effects of NPY in the PVN on thermogenesis are contradictory; both increases and decreases in body temperature were reported (204;205;223). In addition, the thermogenic response to a single injection in the LH fluctuates with the dose of NPY administered, with hyperthermia observed after low doses, and hypothermia after higher doses (204;205).

Considering the diversity in effects of NPY infusion into the PVN and LH/PFA on energy expenditure, chronic elevated NPY in the PVN or LH probably also differentially regulate other parameters related to obesity.

So far, the involvement of different brain regions in the effects of NPY on energy balance was investigated using single (PVN and LH) or repeated injections (PVN) of NPY locally in the brain. In order to understand which brain regions are involved in induction of obesity by increased NPY signaling, it is essential to continuously increase NPY locally in the brain, which is not feasible using infusions of ligands. We have used viral mediated overexpression of NPY to further unravel the specific role of the PVN and LH (including the PFA) in NPY-induced obesity. Recently, it was shown that when NPY is overexpressed with this technique at the adult stage in the whole hypothalamus of mice, obesity is induced (253). In the present study, the long-term effects of NPY overexpression in either the PVN or LH were compared with regard to feeding behavior (including meal patterns), body temperature and locomotor activity. In addition, the site-specific effects of increased NPY signaling on endocrine parameters and body composition were compared.

MATERIAL AND METHODS

Animals

Male Wistar rats weighing 220-250 g were purchased from Charles River (Crl-Wu, Germany). They were individually housed in filtertop cages with *ad libitum* access to food and water. Animals were kept in a temperature and humidity controlled room (21±2 °C) under a 12h/12h light/dark cycle (lights on at 0600 h). All experimental procedures were approved by the Committee for Animal Experimentation of the University of Utrecht, Utrecht, The Netherlands.

Surgical procedures

Rats were anesthetized with 0.1 ml/100 g hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and 0.05 ml/100 g ip dormicum (Hoffman-LaRoche, Mijdrecht, The Netherlands). Transmitters (TA10TA-F40 Data Science International, St Paul, Minnesota, USA) were placed in the abdominal cavity. Rats were left to recover for three weeks.

Seven days after baseline recordings, rats were anesthetized again as described above. Using a stereotax, rAAV-NSE-NPY (n=12, LH-NPY) or rAAV-NSE-empty (used as control) (n=7, LH-contr) was injected bilaterally into the LH (coordinates AP: -3.0 mm from bregma, ML: ±1.6 mm from bregma, and DV: -8.6 mm below the skull). In a second experiment, rats were injected bilaterally into the PVN (coordinates AP: -1.8 mm from bregma, ML: ±0.3 mm from bregma, and DV: -8.1 mm below the skull, rAAV-NSE-NPY n=16 (PVN-NPY), rAAV-NSE-empty n=12 (PVN-contr)). rAAV-NPY and rAAV-empty were a kind gift of M.J. During (New York). Production of rAAV-NPY has been described

previously (339;340). 1 µl of virus (1x10^8 genomic copies) was injected per site over five minutes, after which the needle was kept in place for ten minutes before removal. After each surgery, rats received an injection with 5 mg/kg carprofen (Vericore Ltd, Dundee, United Kingdom).

Data analysis

One week before, until fifty days after viral injections, body weight, food intake, body temperature and locomotor activity were recorded.

Body weight gain and food intake were measured daily at 11.00h. Body temperature and locomotor activity were automatically recorded via the transmitters that send digitized data via radio frequency signals to a nearby receiver. The data were recorded every ten minutes using DSI software (DSI, St Paul, MN). On day 21 and 48 meal patterns were recorded. Food hoppers were weighed automatically and data were send to a computer every 12 seconds for 24 hours. A meal was defined as an episode of food intake with a minimal consumption of 0.5 g chow, and an inter-meal interval of 5 minutes.

Collection of blood and tissues

At day 50 after injection of the virus, rats were decapitated in the morning, trunk blood was collected in heparinized tubes after adding 83 µmol EDTA and 1 mg aprotinin, and immediately placed on ice. Plasma samples were stored at -20 °C until further analysis.

Brains were immediately removed after decapitation, quickly frozen in cold isopentane (-35 °C) and stored at -80 °C. Retroperitoneal, epididymal, mesenteric and subcutaneous white adipose tissue (WAT) was isolated and weighed. Pituitaries, adrenals and the thymus were also isolated and weighed.

Quantitative in situ hybridization

16 µm coronal sections were used for raISH. 33P-labeled antisense RNA probes were made for AgRP, NPY, POMC, MCH and prepro-orexin. raISH procedure has previously been described (345). Expression of AgRP, NPY and POMC in the arcuate nucleus and expression of MCH and prepro-orexin in the LH was quantified using Image J software (National Institutes of Health, Bethesda, Maryland, USA). Expression of NPY was also measured in the PVN and LH to confirm viral-mediated overexpression.

Plasma analysis

Plasma leptin, insulin and corticosterone were analyzed in duplicate using radioimmunoassay kits, (Linco Research, St Charles Missouri, USA for leptin and insulin, ICN Biochemicals, Costa Mesa, California, USA for corticosterone). Plasma adrenocorticotropic hormone (ACTH) was measured in duplicate using a specific rabbit

antiserum directed to the midportion of ACTH, which was kindly provided by Dr G.B. Makara (Budapest, Hungary). Synthetic human ACTH(1-39) (Peninsula Laboratories, Belmont, California, USA) was labeled with ^{125}I and used as tracer (342). Plasma glucose was measured in triplicate using a Glucose/GOD-Perid method (Boehringer Mannheim, Germany).

Statistical analysis

Data are presented as group means ± SEM. Differences in body weight and food intake were assessed using repeated measure analysis. When significant overall interactions were found, post hoc analyses were performed with T-tests. Further statistic analysis was performed with T-tests. Differences were being considered significant at $p < 0.05$.

RESULTS

Verification of AAV injections

Correct injection of AAV-NPY was analyzed by radioactive NPY in situ hybridization. Rats were only included in the study when NPY expression was observed bilaterally in the PVN or LH (Fig. 1), animals with incorrect injections were excluded from analysis (in total, two rats from PVN-contr, three rats from LH-NPY, one rat from LH-contr and seven rats from LH-NPY were excluded, due to unilateral WPRE expression or expression not restricted to the LH).
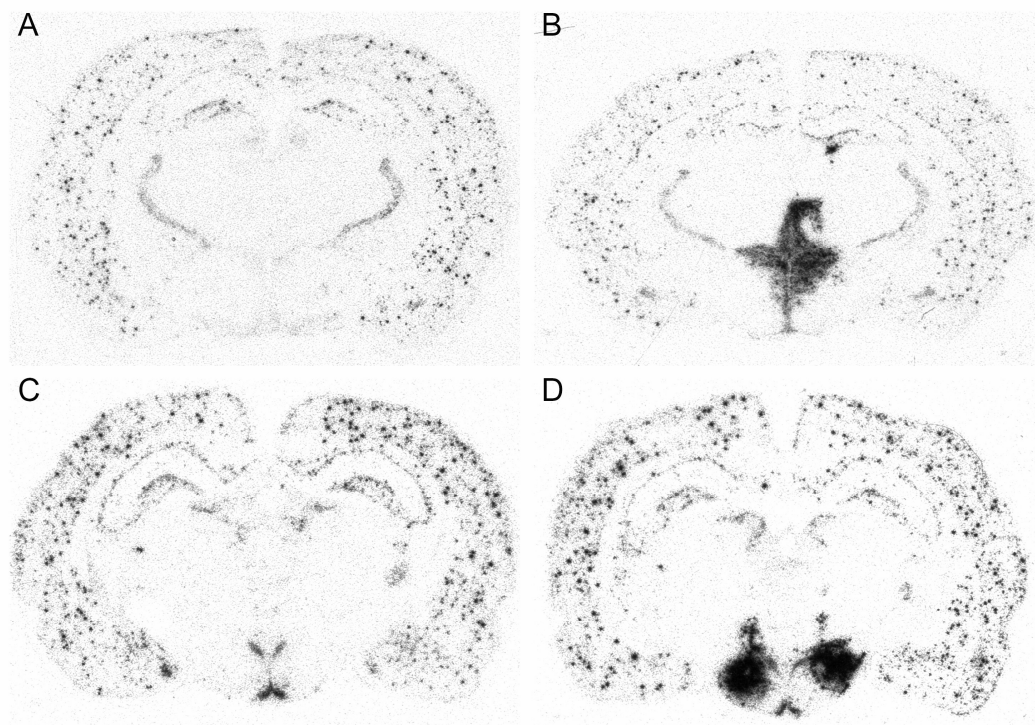
Figure 1: NPY mRNA expression in rats injected with rAAV-empty in the PVN (A) or LH (B), or with rAAV-NPY in the PVN (C) or LH (D).

AAV-NPY induced effects on body weight and food intake

The first days following injection of the viral particles, all groups of rats showed a similar decrease in body weight gain and intake of food and water. All animals recovered within one week after injection. Both PVN-NPY rats and LH-NPY rats showed an increase in body weight gain from day 9 post injection when compared to controls (Figs 2A and B). In the last ten days of the study body weight gain of PVN-NPY rats was not significantly different from PVN-controls, however, body weight gain of LH-NPY rats remained increased until the end of the study. At day 50, PVN-NPY rats had accumulated $167 \pm 11.5\%$ more weight than PVN-contr rats, while LH-NPY rats had accumulated $227 \pm 25.7\%$ more weight than LH-contr rats, which was significantly more than the PVN-NPY rats ($p<0.05$).
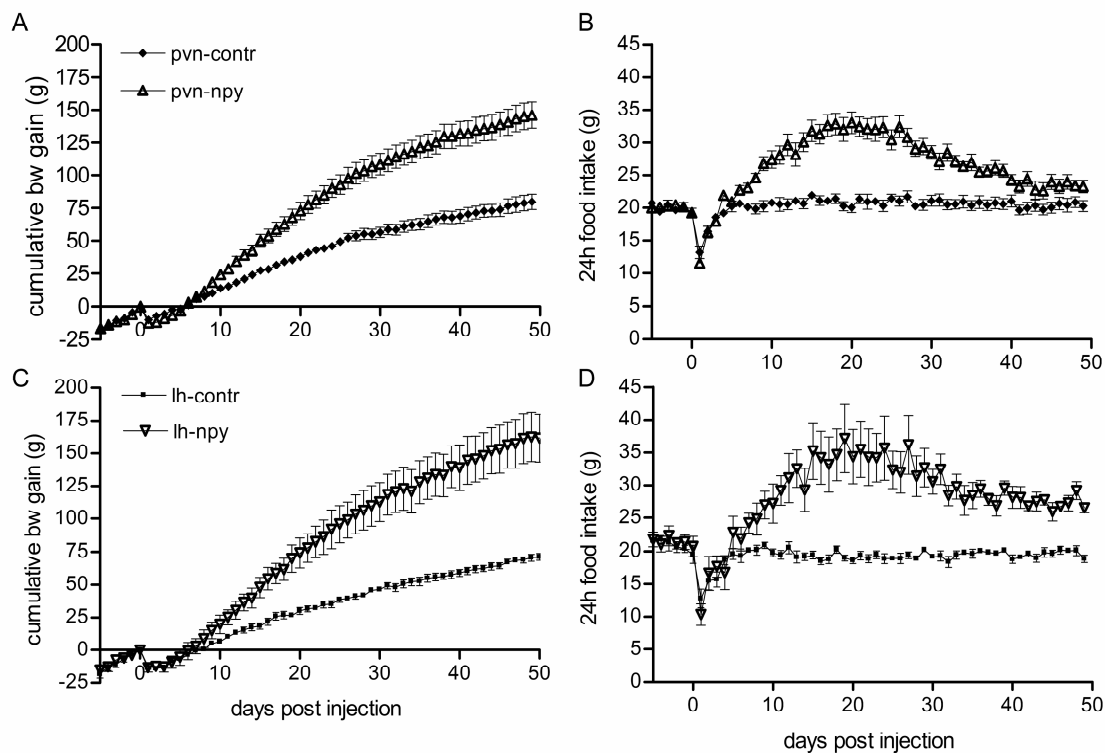
Figure 2: Effects of rAAV-NPY injections in the PVN or LH on cumulative body weight gain (A and C), daily food intake (B and D) compared to rAAV-contr rats.

Daily food intake of both PVN-NPY and LH-NPY rats increased from day 7 after injection until day 15, when it reached a plateau. In PVN-NPY rats, food intake slowly decreased again from day 27 and was similar to controls in the last ten days of the study (Fig. 2C). In LH-NPY rats food intake also decreased from day 27, however, it stabilized at a level that was still significantly increased when compared to LH-contr rats (Fig. 2D), even when food intake was corrected for body weight (day 49: 5.32 ± 0.16 g/100g body weight vs 4.79 ± 0.16 g/100g body weight for LH-NPY vs LH-contr, $p<0.05$).

Daily water intake of PVN-NPY rats followed the food intake pattern, and was significantly increased between day 11 and day 29 when compared to PVN-contr rats (average of 28.2 ± 1.37 ml per day vs 23.1 ± 1.10 ml per day for LH-NPY vs LH-contr, p<0.05). There were no differences observed in water intake of LH-NPY and LH-contr rats (data not shown).
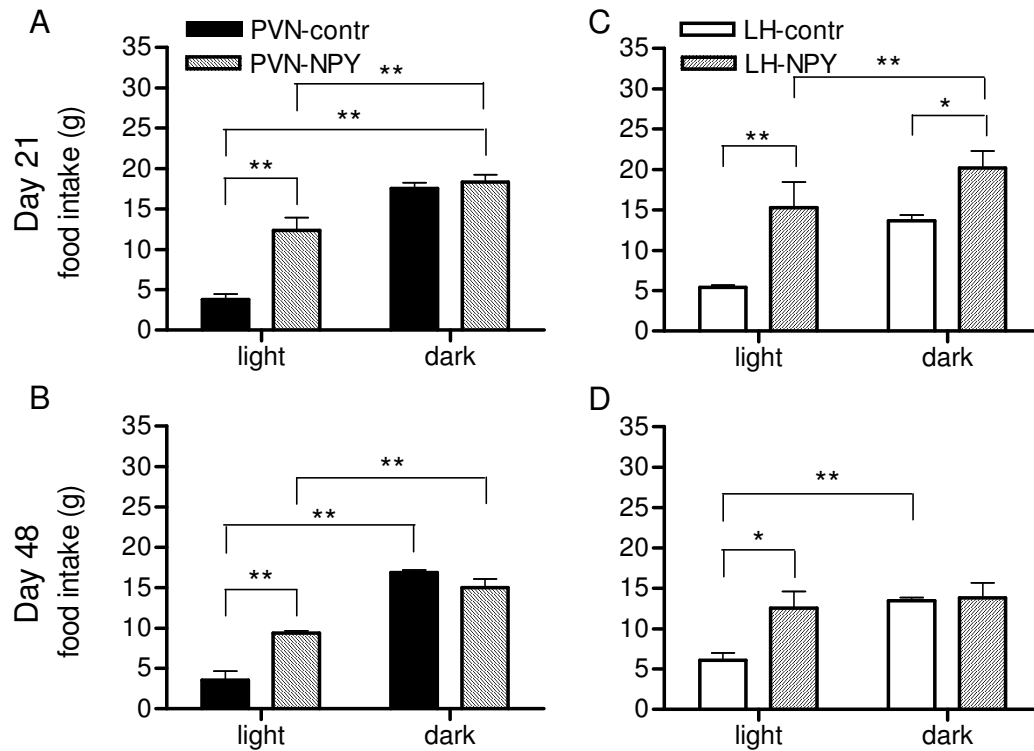
Figure 3: Effects of rAAV-NPY injections in the PVN or LH on dark and light phase food intake on day 21 (A and B) or day 48 (C and D). * p<0.05, ** p<0.01.

AAV-NPY induced effects on meal pattern

As shown above, food intake of rats injected with rAAV-NPY was significantly increased when compared to control rats. To determine whether this increase was due to an increase of meal frequency and/or meal size, meal patterns were analyzed. At day 21, rats that were injected with rAAV-NPY in the PVN showed an increase in light phase food intake, whereas dark phase food intake was similar to control rats (Fig. 3A). Moreover, rats that were injected with rAAV-NPY in the LH showed an increase in both dark phase and light phase food intake (Fig. 3B). At day 48, both PVN-NPY and LH-NPY rats showed a significant increase of food intake only in the light phase (Figs 3C and D).

Both PVN-NPY and LH-NPY rats showed an increased meal frequency in the light phase of day 21 (Figs 4A and B). At day 48, meal frequency of PVN-NPY rats was similar to controls, while LH-NPY rats still showed a significant increase in the light phase (Figs 4C and D). Meal size of rats injected with rAAV-NPY in the PVN was similar to controls both 21

and 48 days after injection (Figs. 4E and G). In contrast, meal size of LH-NPY rats was increased in both the dark and light phase of day 21 and 48 (Figs 4F and H).
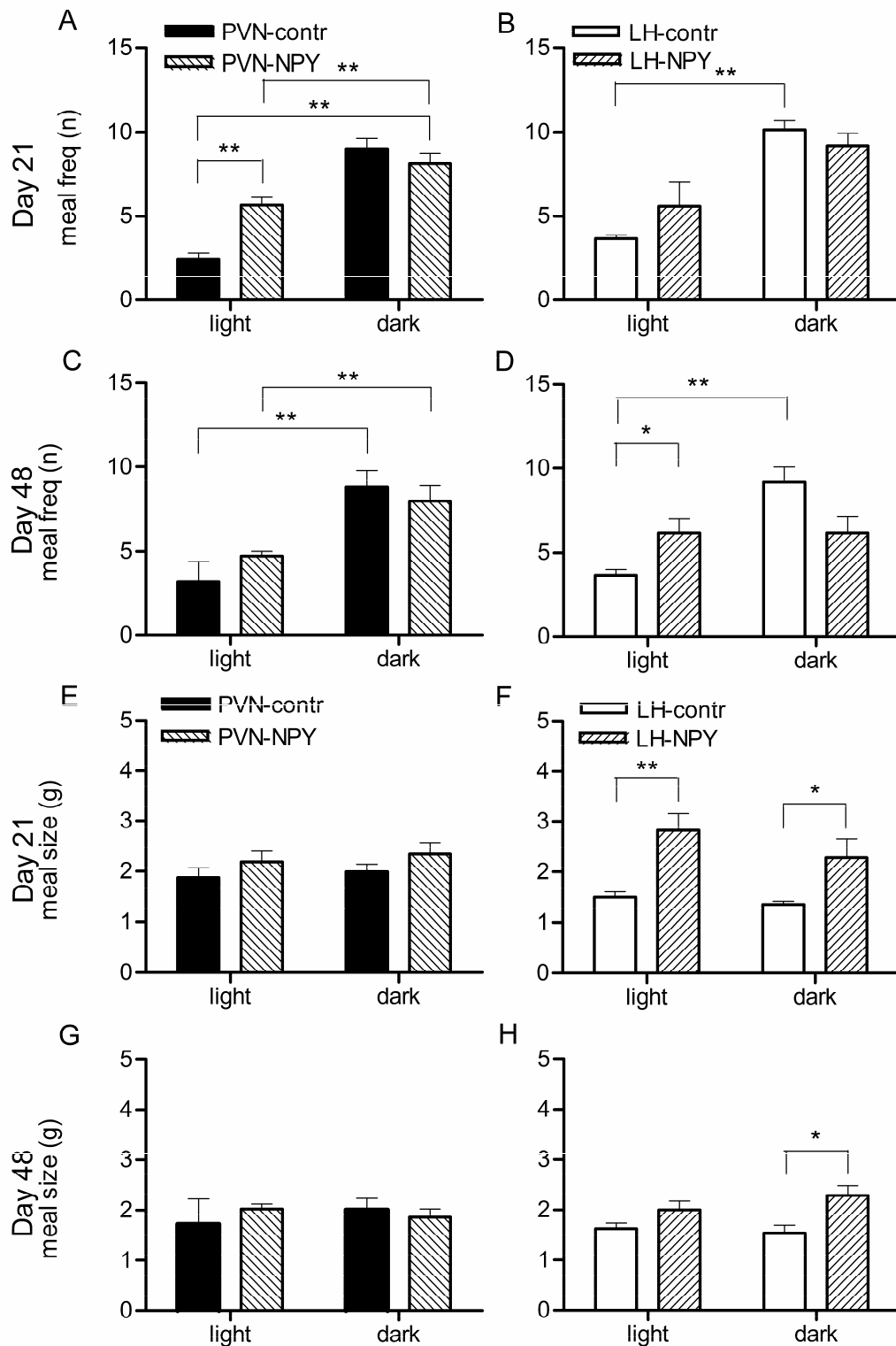

Figure 4: Effects of rAAV-NPY injections in the PVN or LH on meal frequency on day 21 (A and B) or day 48 (C and D) and meal size on day 21 (E and F) or day 48 (G and H). * p<0.05, ** p<0.01.

Although PVN-NPY rats showed an increase in meal frequency and total food intake in the light phase, they still ingested significantly more meals and more food in the dark phase as in the light phase, similar to control rats. In LH-PVN rats on the other hand, both the number of meals and total food intake in the light phase was similar to the number of meals and intake in the dark phase at both time points analyzed (Figs 3 and 4).

AAV-NPY-induced effects on body temperature and locomotor activity

To determine whether NPY overexpression in the PVN and LH has differential effects on energy expenditure, we examined body temperature and locomotor activity on day 21 and 48 following injection of the viral particles.

Dark phase body temperature was significantly decreased in both PVN-NPY and LH-NPY rats when compared to controls on both timepoints analyzed (Table 1). In contrast, both groups of rats injected with rAAV-NPY showed a significant increase in body temperature in the light phase of day 21. On day 48 however, no differences were observed in light phase body temperature (Table 1).

We further analyzed the daily pattern of body temperature (Fig 5). Whereas PVN-NPY rats showed a normal circadian temperature rhythm with clear peaks on both days (despite a decrease in dark phase temperature) (Figs 5A and C), LH-NPY rats showed a flattened pattern on both day 21 and 48 (Figs 5B and D).

Locomotor activity of rats injected with rAAV-NPY in the PVN was reduced only in the dark phase of day 21 and 48, whereas activity of LH-NPY rats was, besides in the dark phase of day 21 and 48, also reduced in the light phase of day 21 (Table 1).

Table 1: Effects of rAAV-NPY injections in the PVN or LH on average body temperature and locomotor activity.

		pvn-contr	pvn-npy	lh-contr	lh-npy
Body temp (º Celsius)					
day 21	light	36.92 ± 0.057	37.08 ± 0.024*	36.99 ± 0.041	37.19 ± 0.063*
	dark	37.78 ± 0.039	37.50 ± 0.036**	37.67 ± 0.031	37.47 ± 0.079*
day 48	light	36.89 ± 0.042	36.89 ± 0.042	36.98 ± 0.072	37.03 ± 0.056
	dark	37.64 ± 0.060	37.26 ± 0.065**	37.63 ± 0.054	37.25 ± 0.132*
Activity (% basal)					
day 21	light	94.6 ± 8.7	89.5 ± 7.6	87.7 ± 4.7	63.1 ± 7.8*
	dark	116.9 ± 9.2	72.5 ± 5.7**	85.1 ± 4.5	55.4 ± 9.1*
day 48	light	75.6 ± 11.3	85.5 ± 11.4	87.5 ± 6.9	76.4 ± 13.3
	dark	102.5 ± 11.4	66.5 ± 5.3**	83.2 ± 6.3	48.8 ± 8.5*

Activity data are presented as percentage of values before injection. *p<0.05, ** p<0.01
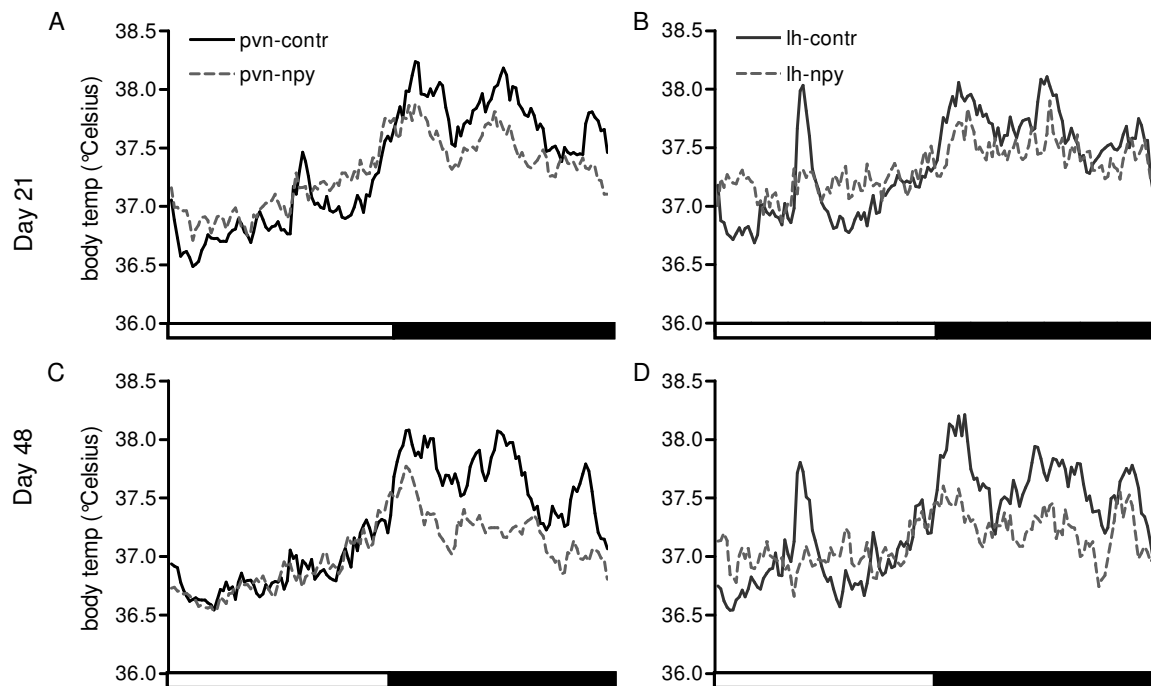
Figure 5: Effects of rAAV-NPY injections in the PVN or LH (data averaged per group) on daily temperature rhythm on day 21 (A and B) or day 48 (C and D).

Table 2: Effects of rAAV-NPY injections in the PVN or LH on endocrine parameters and body composition.

	pvn-contr	pvn-npy	lh-contr	lh-npy
leptin (ng/ml)	4.62 ± 0.50	19.53 ± 2.86**	4.79 ± 0.65	18.86 ± 3.44**
insulin (ng/ml)	4.27 ± 0.67	6.72 ± 0.58*	3.46 ± 0.69	5.99 ± 0.55*
glucose (mmol/l)	5.60 ± 0.11	6.14 ± 0.18*	5.52 ± 0.32	5.73 ± 0.24
cort (μg/dl)	9.70 ± 2.04	20.96 ± 4.62*	3.77 ± 1.13	16.15 ± 8.99
ACTH (pg/ml)	130.81 ± 6.72	311.00 ± 58.61*	104.34 ± 8.50	159.01 ± 40.34
SWAT (%bw)	0.38 ± 0.04	1.15 ± 0.18**	0.46 ± 0.04	1.59 ± 0.20**
AWAT (%bw)	1.98 ± 0.17	4.44 ± 0.45**	1.99 ± 0.09	5.04 ± 0.42**
adrenals (‰bw)	0.09 ± 0.008	0.08 ± 0.055	0.10 ± 0.005	0.09 ± 0.087
thymus (‰bw)	0.91 ± 0.055	0.098 ± 0.088	0.91 ± 0.087	0.79 ± 0.082
pituitary (‰bw)	0.023 ± 0.002	0.018 ± 0.001*	0.024 ± 0.001	0.017 ± 0.001**

SWAT: subcutaneous white adipose tissue, AWAT: abdominal white adipose tissue. * $p<0.05$, ** $p<0.01$

AAV-NPY-induced effects on endocrine parameters and body composition

Table 2 summarizes the effects of NPY overexpression in the PVN and LH on endocrine parameters and body composition.

Subcutaneous and abdominal fat pads and plasma concentrations of leptin and insulin were significantly increased in both PVN-NPY and LH-NPY rats. Whereas plasma glucose levels were normal in rats injected with rAAV-NPY in the LH, rats injected in the PVN displayed increased concentrations of plasma glucose when compared to controls.

Figure 6: Effects of rAAV-NPY injections in the PVN or LH on mRNA expression of POMC (A), AgRP (B), NPY (C) in the Arc, and orexin (D) and MCH (E) in the LH. * p<0.05, ** p<0.01.

Plasma concentrations of both corticosterone and ACTH were increased in PVN-NPY rats when compared to PVN-contr rats, but not significantly different between LH-NPY and LH-contr rats. No differences were found in the weight of the thymus or adrenal glands, but the weight of the pituitary gland was significantly decreased in both PVN-NPY and LH-NPY rats.
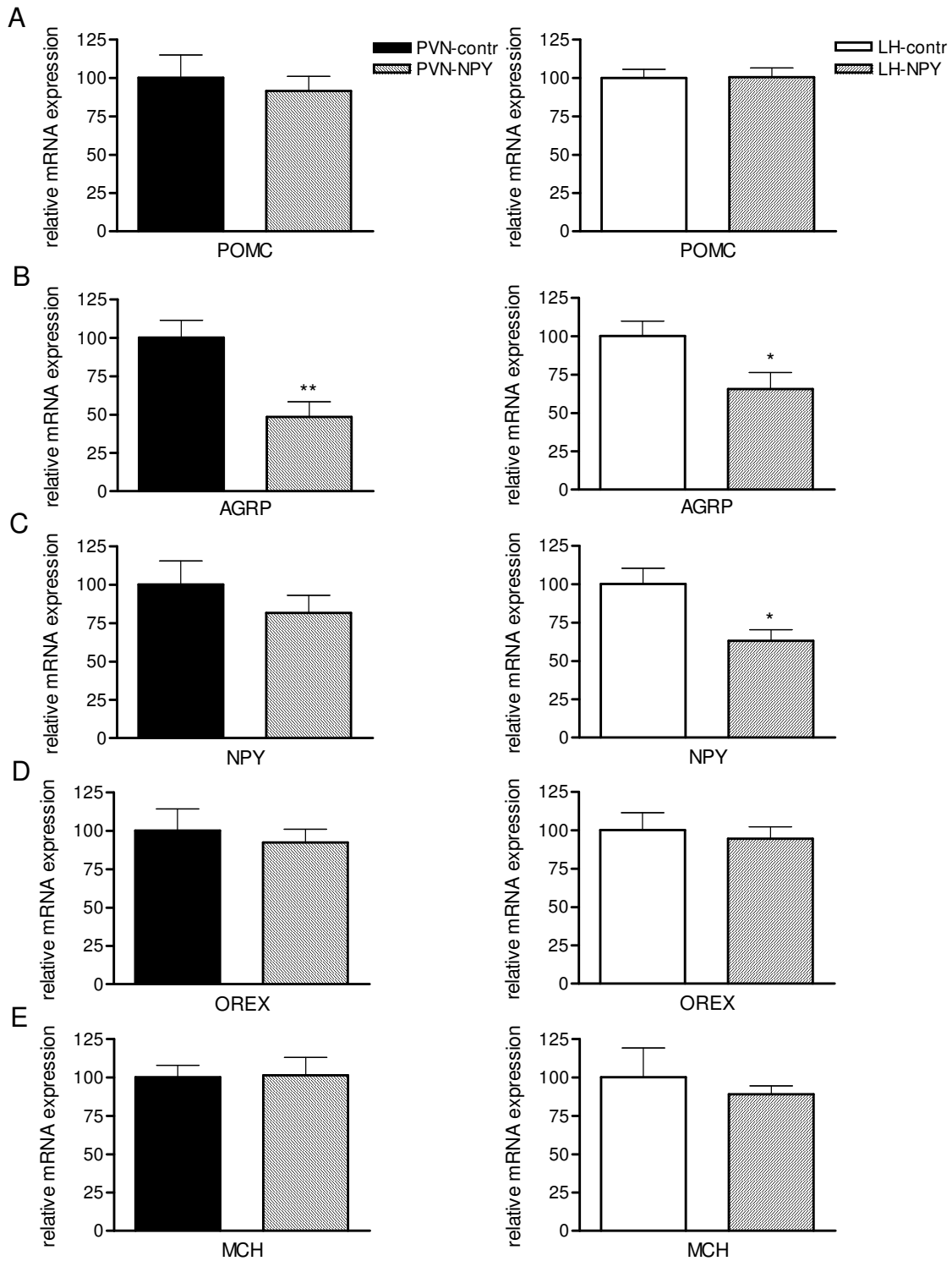
AAV-NPY-induced effects on neuropeptide expression in the Arc and LH

Fifty days after injection of the viral particles, mRNA expression of NPY, AgRP and POMC in the arcuate nucleus, and orexin and MCH was measured by raISH (Fig. 6). When compared to control rats, rats injected with rAAV-NPY in both the PVN and LH showed a similar reduction of AgRP mRNA in the Arc, but no change in POMC mRNA (Figs 6A and B). In LH-NPY, but not PVN-NPY rats, also expression of NPY mRNA was significantly reduced (Fig. 6C). Expression of orexin and MCH in the LH were not altered in rats injected with rAAV-NPY when compared to rats injected with rAAV-contr (Figs 6C and D).

DISCUSSION

Both PVN-NPY rats and LH-NPY rats developed obesity, accompanied by an increase in food intake and concentrations of leptin and insulin, and a decrease in dark phase body temperature and locomotor activity. However, further analysis revealed clear differences in how obesity was induced when NPY was chronically overexpressed in these brain regions. While in PVN-NPY rats food intake was elevated by a specific increase in the number of meals eaten in the light phase, in LH-NPY rats also meal sizes in dark and light phase were increased. Moreover, in LH-NPY rats, but not PVN-NPY rats, circadian rhythmicity with regard to food intake and body temperature was lost. A comparison of the effects observed after injection of AAV-NPY in the PVN and LH is presented in table 3.

NPY overexpression in the PVN or LH resulted in an increase in food intake, which was similar and maximal between day 15 and 27 post injection. Subsequently, PVN-NPY rats reduced their food intake until both food intake and body weight gain was comparable to controls. However, in LH-NPY rats, despite a small decrease, food intake and body weight gain remained elevated when compared to controls.

In PVN-NPY rats, eventually compensatory mechanisms are effective to normalize food intake to control levels, possibly reflecting a new level of body weight or adiposity (Chapter 5). In LH-NPY rats however, food intake remains increased and can not be compensated for completely, despite reduced expression levels of NPY and AgRP mRNA in the Arc. Electrical stimulation of the LH is known to initiate a feeding motor program even in satiated rats (365). In addition, NPY terminals in the LH innervate MCH and orexin neurons (366;367), which project to areas that are important for salivation, arousal and locomotor activity (30;368). NPY signaling in the LH might therefore be more involved in

aspects leading to feeding, which could be less sensitive to compensatory effects such as increased levels of leptin and decreased expression of AgRP and NPY in the Arc. Indeed, although food entrained rats show an increased Fos expression and multiple unit activity in the LH and PVN in anticipation to food, only the food-entrained rhythm of the LH persists after fasting (369;370). This suggests that the LH, rather than the PVN, is involved in aspects prior to the initiation of feeding.

Table 3: Comparison of the effects observed after injection of AAV-NPY in the PVN and LH.

	day 21		day 48	
	PVN	**LH**	**PVN**	**LH**
body weight gain	↑	↑	↔	↑
fat %			↑	↑
food intake	↑	↑	↔	↑
meal size	↔	↑	↔	d↑
meal freq	↑	↔	↔	l↑
water intake	↑	↔	↔	↔
body temp	l↑, d↓	l↑, d↓	d↓	d↓
locomotor act	d↓	↓	d↓	d↓
body temp rhythm	↔	↓	↔	↓
feeding rhythm	↔	↓	↔	↓
plasma glucose			↑	↔
HPA axis			↑	↔

l: light phase, d: dark phase

Analysis of meal patterns revealed that, in the period when daily food intake was maximal, PVN-NPY rats only increased food intake in the light phase, whereas LH-NPY rats ingested more food in both the light and the dark phase when compared to controls. It has been reported that a 4 hour infusion of NPY in the third ventricle increases meal frequency (195). In addition, a single injection of NPY in PVN increases the size of the first meal after injection rather than meal numbers (220;371), whereas a single injection of NPY in the PFA increases food intake due to both size and frequency of meals (227). This is partly consistent with the data described here. We have found that the increase in feeding of PVN-NPY rats was due to an increase in the frequency of normal sized meals, while LH-NPY rats consumed larger meals. Thus, although short-term effects of NPY on food intake may only increase the initiation of meals via signaling in the PVN, effects of long-term overexpression may also involve NPY signaling in the LH, thereby also altering meal size.

Thus far, acute effects of NPY in the PVN on body temperature were contradictory (204;205;223), and effects of NPY in the LH depend on the dose injected (204;205). However, after chronic overexpression of AAV-derived NPY, both rats injected in the PVN and rats injected in the LH show relative hypothermia in the dark phase and (temporal) relative hyperthermia in the light phase when compared to controls. It has been hypothesized

previously that, although NPY signaling in the LH/PFA is important for energy intake, it is not involved in energy expenditure, since body temperature, locomotor activity and respiratory quotient are not altered following an acute injection of NPY in the PFA (204;205;224). Nevertheless, reductions in both dark phase body temperature and locomotor activity were observed in PVN-NPY and LH-NPY rats. In addition, LH-NPY rats also showed a transient reduction in light phase locomotor activity, despite an increased body temperature during this phase of the day. The effects on temperature and activity levels in LH-NPY rats may largely be caused by an increased NPY signaling in the LH, and not by an increase of NPY in the PFA. However, also one rat that had an overexpression of NPY limited to the PFA showed these reductions. Although we can not exclude that this was caused by release of viral-derived NPY to the LH, it is possible that both the PVN and the LH are involved in the effects of NPY on long-term energy expenditure.

Despite the changes in average body temperature, PVN-NPY rats display a normal circadian rhythm, while this is not true for LH-NPY rats. In these rats, although some oscillation is still observed, the overall amplitude of the circadian temperature pattern is markedly reduced. Furthermore, despite the fact that PVN-NPY rats increased only their light phase food intake, they still ingested more food during the dark phase, which is the normal time for a rat to eat. LH-NPY rats on the other hand consume as much food in the light phase as in the dark phase, by decreasing the number of meals consumed in the dark phase and increasing meal frequency in the light phase. This may be explained by the fact that either ICV or LH injections of NPY induce wakefulness (372), probably via orexin neurons. Interestingly, the suprachiasmatic nucleus, which drives the circadian rhythm of feeding activity, is hypothesized to accomplish this via projecions to the orexin and MCH neurons of the LH (373). A continuous stimulation by increased NPY signaling to the orexin neurons and thus the motor circuits involved in feeding behavior would therefore explain the increased anticipation to eat as well as the time spent feeding. Together with the blunted temperature pattern of LH-NPY rats, this provides evidence for a role of NPY signaling in the LH in daily rhythms of both food intake and body temperature.

One could argue that, due to the increase in food intake, also water intake should increase. Indeed, acute injections of NPY in the PVN have been reported to result in an increase in water intake, which is not observed after injections of NPY in the LH (374). In line with this, we observed a small increase in water intake in PVN-NPY rats, whereas LH-NPY rats drank the same amount of water as controls, despite their increase in food intake. It can therefore be concluded that the increased drinking behavior of PVN-NPY rats is not simply a reflection of increased feeding behavior. This is strengthened by the fact that the drinking response is also observed in the absence of food when NPY is injected into the PVN (220).

NPY is known to have a stimulating effect on the HPA axis (203;354) and in turn an increase in HPA axis activity is associated with obesity (355). Therefore, we also investigated the effects of NPY overexpression on the HPA axis. Earlier we have found that AAV-induced NPY overexpression in the PVN did not increase HPA axis activity 23 days after injection (Chapter 4). However, 50 days after injection, we observed an increase in plasma concentrations of ACTH and corticosterone in rats that were injected with AAV-NPY in the PVN. Rats that were injected with AAV-NPY in the LH however did not alter the activity of the HPA axis. This suggests that the effects of NPY on the HPA axis are specifically regulated by the PVN. This is in line with the fact that CRF is produced in neurons of the PVN, but not in the LH (375). Nevertheless, the effects on the HPA axis become evident at a later time-point than the effects on food intake.

In conclusion, these results show that, although chronic NPY administration results in a common obese phenotype, there are region-specific effects of NPY overexpression. Whereas both areas can alter energy expenditure in a similar manner, NPY signaling in the PVN is involved in the initiation of food intake and activation of the HPA axis. Increased NPY signaling in the LH, on the other hand, increases meal frequency and size, and can alter circadian patterns to a status where an animal is constantly ready to eat, thereby equalizing light and dark phase food intake and body temperature. This suggests that the NPY system differentially regulates energy intake and energy expenditure in the hypothalamic nuclei, which together adjust energy balance.

Chapter 7

Interfering with neuropeptidergic systems; a
comparison of strategies

INTERFERING WITH NEUROPEPTIDERGIC SYSTEMS; A COMPARISON OF STRATEGIES

The central regulation of energy balance is a complex process, composed of multiple interacting pathways. To increase the knowledge of systems and brain areas involved in the regulation of energy balance, various strategies have been used. Already in the fifties lesion studies revealed that the hypothalamus plays an important role in the control of body weight (2-4). However, not until 1994, when the leptin gene was cloned (376), knowledge about the neural systems involved in the central regulation of energy balance expanded. Since then, a lot of genes have been found to play a role in the regulation of energy homeostasis and an array of genetic obesity models has become available. In addition, to gain more insight in how and where neuropeptidergic systems control energy balance, administration of agonists or antagonists either ICV or local has been used. In this thesis, it was aimed to further clarify the contribution of MC and NPY signaling in distinct brain areas in the development of obesity.

Despite the enormous amount of information that has been obtained following the various strategies mentioned here, there are some drawbacks one has to keep in mind (Table 1), especially when investigating the local role of neuropeptides. Below, the most important advantages and disadvantages of the different strategies used in the exploration of energy balance are discussed.

Table 1: Characteristic of the various strategies used to unravel the regulation of energy balance.

	germline genetic	conditional genetic	pharm infusion	viral vector
certainty normal development	-	+/-	+	+
local interference	+/-	+/-	+/-	+/-
long-term interference	+	+	-	+
stable gene expression	+	+	-	+

GENETIC INTERFERENCE

Germline transgenic and knock out studies are widely used to gain information about the contribution of a gene to energy homeostasis. Indeed, the technique is ideal to investigate the overall function of a gene. In addition, long-term absence of a gene can be studied. Examples of genes that are deleted to study their function in the control of body weight are leptin, NPY and AgRP (152;235;376).

However, conventional knock out strategies result in the deletion of a gene in all cells. Besides the fact that this prevents the exploration of site-specific effects of a gene, it

also does not represent a normal physiological situation. Furthermore, alterations in gene expression due to genetic interference can have secondary effects. Leptin for example is required for a normal development of arcuate projection pathways (254;255). Mice lacking a functional gene encoding leptin or the leptin receptor have therefore an altered neural system, which can exaggerate the effects of reduced leptin signaling. Moreover, the brain shows a massive plasticity during development, which is shown by the fact that AgRP/NPY neurons can be ablated during development, while they are required for normal feeding in adult mice (155;156). Due to this plasticity, it is possible for other systems involved in energy balance to compensate for the loss of a gene in development. This can explain why NPY$^{-/-}$ mice and AgRP$^{-/-}$ mice are relatively normal, while alterations in NPY or MC signaling in adult wild-type mice does have obvious effects.

More or less the same problems are met in transgenic studies. Ectopic overexpression of agouti results in a specific obesity phenotype (139). Nevertheless, it is possible that a part of these symptoms is due to an altered development. Furthermore, since A^y mice overexpress agouti both centrally and peripherally, it cannot be determined where and how agouti acts in the development of obesity.

Besides, one has to keep in mind that the effects of genetic alterations depend on the background of the mouse strain used (241;242;377;378). The 129 strain for example is much less sensitive to obesity than the C57Bl/6J (241;242). As a result, varying or even opposite findings can be obtained when the function of a gene is analyzed on different backgrounds. Indeed, depending on the background, deletion of the Y2 receptor has been reported to either increase or decrease food intake and body weight gain (185;249).

An improvement of genetic interference came with the introduction of conditional knockouts, which are used to delete a gene in a particular organ, cell type, or stage of development. The most widely used method is the Cre-loxP recombinase system which is for instance used to generate mice with a hypothalamic-specific deletion of the Y2 receptor (249). It is also used to create MC4$^{-/-}$ mice in which MC4 expression can be rescued specifically in the PVN by crossing the mice with *Sim1*-Cre transgenic mice (379). Another example of a conditional knock out strategy is the use of the Tet-On system, which allows inducible regulation of a gene (380), and is used to study the blockage of NPY expression in the adult mouse (232). Nevertheless, also in these models developmental changes can not be excluded.

PHARMACOLOGICAL INTERFERENCE

In contrast to the genetic modification of a gene, pharmacological administration of neuropeptides does not interfere with development. Moreover, ligands can be injected locally in the brain, allowing the investigation of the role of specific areas in a neuropeptidergic system. However, although ICV infusions for one week are feasible, this is

not true for local infusions. Thus, also with a pharmacological approach it is not possible to explore the long-term contribution of neuropeptide signaling in a specific target site in energy balance, i.e. in the development of obesity.

AAV-MEDIATED INTERFERENCE

In the present experiments, recombinant adeno-associated (rAAV)-induced overexpression of neuropeptides was used to further understand their local role in the central regulation of energy balance. rAAV-mediated gene expression overcomes the major shortcomings of both genetical and pharmacological strategies. As shown in table 1, rAAV-mediated gene expression can result in a stable long-term expression of the transgene, in a local area, without altering developmental processes.

Also rAAV studies have some disadvantages. Firstly, it is questioned whether the observed effects cannot be attributed to neuronal transport of either the virus itself or the transcribed product in the infected neuron. However, no agouti staining could be observed in target areas of the PVN, as the brain stem, after injection of rAAV-agouti, which could indicate anterograde transport of the transcribed transgene (chapter 2). In addition, no viral gene expression was observed in cell bodies of the Arc, which could indicate retrograde transport of the virus. Therefore, we can assume that neuronal transport to areas outside the injection area did not contribute to our results. To be absolutely sure that the transgene is only expressed in the target area, one could choose to make use of an area-specific promoter, as for example the *Sim1* promoter for PVN specific expression.

Secondly, it is argued that injection of rAAV infects multiple subsets of neurons, including neurons that normally do not express the transgene. To bypass this issue, gene-specific promoters could be used.

COMBINED INTERFERENCE

Table 1 shows that the strategies have overlapping advantages and disadvantages. Obviously, it depends on the research question which of the approaches will result in the most clear-cut answers. Probably the best models to further investigate long-term, site specific effects of neuropeptides are a combination of genetic models and interference with rAAV. An example of such a combination is used by Coppari *et al*. They have reexpressed the leptin receptor of FLPe-reactivatable leptin receptor null mice by injecting rAAV-FLPe specifically in the Arc (381). This resulted in an improvement of hyperinsulinemia and a modest decrease in body weight (381). Nevertheless, these animals do lack the leptin receptor during development, which is known to alter projection pathways from the Arc (254;255). To investigate the role of a gene in a specific area, it would therefore be better to generate an inducible knockout, which has a normal development, and with the use of rAAV, locally delete the gene.

Another way to explore the role of decreased neuropeptide signaling in the brain is to use rAAV-mediated RNA interference (382). An advantage of this method is that the gene of interest will not be silenced completely, which might mimic better the genetic variation in the human population. Especially combined with gene-specific or site-specific promoters this would be an excellent manner to further unravel the mechanisms regulating energy balance.

Chapter 8

Summary and general discussion

SUMMARY AND GENERAL DISCUSSION

DISSOCIATION OF THE MC AND NPY SYSTEM IN ENERGY BALANCE

There are many similarities between NPY and AgRP. Both neuropeptides are produced in the same neurons of the Arc and thus released in the same projection areas of the Arc (7). Both are orexigenic neuropeptides that respond similar to leptin treatment or fasting (383-385). Furthermore, both AgRP$^{-/-}$ mice and NPY$^{-/-}$ mice display a relatively normal phenotype (152;235;238;243). Moreover, even deletion of both genes does not have a clear effect on food intake or body weight, suggesting that neither AgRP nor NPY is required for a normal energy balance (152).

Nevertheless, there are differences between the two genes. Whereas an acute central injection of NPY has a direct, short lasting effect on food intake, the effects of AgRP are, depending on the time of injection, slower and can last for seven days (113;114;193;194). Furthermore, absence of the leptin receptor results in increased NPY levels in the Arc, while AgRP levels remain normal (386). Also the response to stress differs, with an upregulation of arcuate NPY levels, but a downregulation of AgRP after a foot shock (387).

Thus, although these neuropeptides seem to have a similar overall function, more careful analysis results in clear differences of the two systems. The AAV-based approach used in the experiments described in this thesis forms an excellent way to further dissociate the function of AgRP and NPY in energy balance.

Development of obesity

One week infusions of both NPY and AgRP are known to result in obesity, caused by hyperphagia and a reduction in energy expenditure (119-121;203). In both systems, the effects on energy expenditure are, at least partly, independent from food intake, as shown by pair-fed studies. Although restricted feeding after NPY or AgRP infusions prevents animals from getting heavier than controls, fat percentage does increase (119-121;203). This indicates that obesity is caused by both an altered energy intake and altered energy expenditure.

Energy intake

As observed after acute ICV injections of NPY or AgRP, AAV-NPY injections resulted in a larger effect on food intake than injection of AAV-Agouti, which results in inhibition of MC receptor activity (Table 1). Furthermore, whereas both increased NPY and decreased MC signaling in the PVN play a role in the regulation of food intake, in the LH only increased NPY signaling has a striking effect on food intake. Reduced MC signaling in the LH does not affect intake of normal chow at all (Chapters 2 and 6). This is consistent

with data from acute injections of MC4 antagonists in the LH, which have shown that reduced MC signaling in the LH does not affect food intake (123).

NPY is thought to affect the appetitive phase of food intake, but not the consumatory phase, since acute ICV injections of NPY only increase meal frequency, without affecting meal size (196;197). Also AAV-mediated chronic overexpression of NPY in the PVN resulted in an increase in meal frequency, while meal size remained normal (Chapter 6). Nevertheless, increased NPY signaling in the LH did, besides increasing meal frequency, also increase meal size (Chapter 6). This suggests that, at least in chronic overexpression, NPY does affect both the appetitive and consumatory phase, although by distinct hypothalamic nuclei.

Meal patterns of rats injected with AAV-agouti remain to be investigated. Based on acute ICV injections of MC agonists however it can be expected that MC signaling only plays a role in the consumatory phase of appetite, since MTII is not able to influence meal frequency, while it does reduce meal size (388). Another argument in favor of this theory is that AgRP injections in mice and rats only increase food intake when meal onset is triggered (81;127), showing that AgRP is not involved in the initiation of food intake. Nevertheless, satiated rats that are fed a high fat, high sucrose diet do increase the intake of fat and chow in the light phase after an ICV injection of AgRP (la Fleur, unpublished observations). This suggests that in some circumstances AgRP can trigger meal onset.

Table 1: Site-specific effects of AAV-Agouti and AAV-NPY fifty days after injection.

	AAV-Agouti			AAV-NPY	
	PVN	LH	DMH	PVN	LH
max 24h FI *	117	100	110	154	180
meal size	nd	nd	nd	=	↑
meal freq	nd	nd	nd	↑	↑
50d bwgain *	145	100	122	167	227
DIO	no	yes	no	nd	nd
compensation	no	no	no	yes	partly
WAT *	nd	nd	nd	227	270
leptin *	nd	nd	nd	419	393
EE	nd	nd	nd	↓	↓

DIO: diet induced obesity, WAT: white adipose tissue, EE: energy expenditure, * % control rats, nd: not determined

Furthermore, increased NPY signaling in the PVN and LH mainly increased light phase food intake, having no (PVN) or only limited (LH) effects on dark phase intake (Chapters 4 and 6). It would be interesting to expose AAV-NPY injected rats to a feeding schedule where they are only allowed to eat in the dark phase, to investigate to what extent light and dark phase food intake contribute to the development of obesity.

This also remains to be explored for chronically decreased MC signaling by injection of AAV-agouti. Nevertheless, as described above, injections of MC4 antagonists typically have a larger effect on food intake when meal initiation is triggered, for instance by fasting or dark onset (81;127). It might be expected therefore that increased AgRP or agouti signaling would increase dark phase intake more than light phase intake.

These results together suggest that the NPY system, especially in the PVN, is mainly involved in the initiation of feeding, and therefore also feeding in the light phase, whereas the melanocortin system is only able to alter meals that are normally eaten, thus, only meals in the dark phase. The increased food intake in both the dark and light phase after NPY overexpression in the LH may be explained by connections of MCH and orexin neurons in the LH with reward centers in the brain, i.e. the nucleus accumbens (60;61), resulting in an increase in rewarding aspects of food intake and thus an increase in meal size.

Energy expenditure

Increased NPY signaling, induced by AAV-NPY, reduces energy expenditure via both the PVN and LH, as shown by a reduced body temperature and locomotor activity in the dark phase (Chapter 6). These effects are independent from food intake, since rats that are prevented to increase their food intake after AAV-NPY injections show a similar reduction in body temperature and activity (Chapter 4). In addition, the effects on body temperature seem not to be entirely related to activity levels of the rat, since locomotor activity is reduced in the light phase of LH-NPY and PVN-NPY rats, while body temperature in that phase is increased (Chapter 6). However, the increased body temperature may also be explained by an increase in light phase food intake.

Aged AgRP$^{-/-}$ mice display an increased energy expenditure, as shown by an increased locomotor activity, body temperature and metabolic rate (154). In line with this, AAV-mediated long term overexpression of POMC increases UCPI mRNA in obese rats, which also suggests that increased MC signaling increases thermogenesis (133;324). In addition, chronic reduction of MC signaling by AgRP infusions reduces oxygen consumption and levels of UCPI (119;120). Thus, there is strong evidence supporting a role for the MC system in the regulation of energy expenditure. Nevertheless, it should be further unraveled which hypothalamic areas are responsible for the effects on energy expenditure by reduced MC signaling.

Endocrine parameters

As expected by the increased body weight gain of rats injected with AAV-NPY in the PVN or LH, the amount of white adipose tissue (WAT) was also increased, as well as concentrations of leptin and insulin (Chapter 6, Table 1). Interestingly, the increase in leptin and insulin of rats injected with AAV-NPY in the PVN preceded the increase in food intake

and weight gain (Chapter 4). This suggests that there was a direct effect of NPY signaling in the PVN on secretion of leptin and insulin.

From the increased body weight of rats injected with AAV-agouti in the PVN or DMH, it might be expected that WAT, leptin and insulin are increased. Although this remains to be further explored, it is known that chronic ICV injections of an MC4 antagonist increase WAT and leptin (116). Furthermore, despite the fact that the effects of NPY on food intake are stronger than those of the MC antagonist HS014 (121), it has been shown that blockade of the MC system results in larger fat accumulation than NPY injections, when increased food intake is prevented. Nevertheless, effects on levels of leptin and insulin are larger after NPY than HS014 in pair-fed rats.

In conclusion, both increased NPY signaling and decreased MC signaling have effects on endocrine parameters that are partly food-independent. Although we have shown that the direct effects of NPY on leptin and insulin secretion can be mediated by the PVN, future studies are necessary to investigate whether other hypothalamic areas also contribute to the increased levels of leptin and insulin observed after chronic infusions of NPY. In addition, the hypothalamic sites that are responsible for the effects of MCs on endocrine parameters also remain to be investigated.

Compensatory pathways

AAV-mediated NPY overexpression in the PVN eventually resulted in the activation of compensatory mechanisms, which normalize food intake and body weight gain (Chapter 5). This is only partly observed after AAV-NPY injection in the LH (Chapter 6), and not after one week infusions of NPY (203;212;214), where food intake and body weight gain remain elevated when compared to controls. Interestingly, plasma levels of leptin are equally increased in animals injected in the PVN and LH. In addition, a similar reduction in AgRP expression in the Arc was observed in both groups of animals, whereas POMC levels remained normal. Moreover, AAV-NPY injections in the LH, but not the PVN resulted in a decrease in NPY mRNA expression in the Arc. Consistently, mice where NPY is disrupted have been found to display an increase in AgRP mRNA levels in the Arc and no changes in POMC expression (243). Together these data suggest that the melanocortin system reacts to changes in NPY signaling by adapting AgRP levels, whereas POMC levels are not affected. This could be due to the fact that increased POMC would not only stimulate the MC system, but, via release of β-endorphin also the opioid system, which can increase food intake (389). The reduction in MC signaling by AgRP can explain both the normal phenotype of NPY^{-/-} mice and the normalization of food intake in rats overexpressing NPY in the PVN.

Nevertheless, counter-regulatory adaptations as increased leptin levels or decreased AgRP expression can not account on their own for the compensation in food intake observed in rats injected with AAV-NPY in the PVN, since these changes are already present three

weeks after injection, when food intake is still maximal (Chapter 4). The pair-fed studies described in chapter 5 indicated that increased NPY signaling in the PVN alters body weight set-point. Only when this new set-point is reached, other compensatory pathways become effective, which, possibly together with the decreased AgRP levels are able to normalize food intake. Possible candidates for these secondary activated pathways are the MC receptors. NPY$^{-/-}$ mice are known to have an increased feeding response to AgRP, suggesting that the MC system is more sensitive to the effects of AgRP (166). A similar alteration in postsynaptic signaling of the MC receptors could very well play a role in the normalization of food intake in PVN-NPY rats. Nevertheless, also pathways outside the MC system could accomplish compensation. For example, changes in catecholaminergic neural signaling could result in a reduction of NPY-induced food intake, because both peripheral injections of amphetamine and dopamine injections in the PFA have been shown to attenuate the NPY-induced feeding response (390;391). In addition, NPY-induced feeding is also mediated in part via the opioid system (392) and it is known that opioid antagonists advance meal termination (393). Although it is known that NPY-induced feeding via the PVN depends on the opioid system (394), it remains to be determined whether also NPY-induced feeding via the LH depends on the opioid system.

However, compensatory pathways are not able to completely normalize food intake, meal size and weight gain of rats that display an increased NPY signaling in the LH. It is hypothesized that the LH is more involved in the anticipatory aspects leading to feeding than in feeding itself, since it projects to areas involved in arousal, activity and salivation (30;368). These pathways may not be very sensitive to compensations in the Arc, such as decreased AgRP and NPY expression, explaining the sustaining effects on food intake in rats injected with AAV-NPY in the LH.

Furthermore, the compensatory pathways that are responsible for the reduction in food intake in the PVN-NPY rats are not able to reduce energy expenditure, since locomotor activity and body temperature remain reduced until the end of the experiment (Chapters 5 and 6). This indicates that the effects of increased NPY signaling on food intake and energy expenditure are mediated by separate pathways. Indeed, NPY reduces thermogenesis by inhibiting the sympathetic outflow to brown adipose tissue, a pathway that does not affect food intake (208).

In contrast to the adaptations observed after altered NPY signaling, neither expression of NPY and AgRP in the Arc, nor expression of MCH and orexin in the LH is changed after viral-induced chronic overexpression of agouti in the PVN, LH or DMH (Chapter 2). This may explain why food intake and body weight gain remained stable after injection of AAV-agouti. In line with these data, a reduction in AgRP expression, or reduced MC signaling is not able to induce compensatory changes in arcuate NPY expression. Genetic changes in MC signaling, as in MC4$^{-/-}$ mice and AgRP$^{-/-}$ mice, or ectopic expression

of agouti, do not alter expression of NPY and POMC mRNA in the Arc (43;152). Moreover, the NPY-induced feeding response of MC4$^{-/-}$ mice is normal, indicating that NPY signaling is not altered in these mice (159). In addition, pharmacological blockade of MC receptors by infusion of SHU9119 does not affect neuropeptide levels in the Arc (305).

Thus, although the MC system does play a role in the compensation of altered NPY signaling, the NPY system is unable to adapt to changes in MC signaling. This suggests that the MC system is crucial for the regulation of energy balance. Indeed, the availability of both agonists and endogenous inverse agonists provides a system that can result in a very tight regulation of homeostasis.

Food preference

Mice lacking the MC4R display an increased intake of high fat food. This can be due to a deficit in sensing the increased caloric density of fat, but also by an increased preference for fat (palatability). In a paradigm where animals can choose between a high fat, high protein and high carbohydrate food source, MTII administration specifically reduces the intake of high fat. This is absent in MC4$^{-/-}$ mice (169). In addition, obese Ay mice have an enhanced preference for high fat in a free choice model (146). Although these data indicate that reduced MC signaling indeed increases preference for fat, they do not reveal where in the brain the MC system affects the rewarding aspects of food palatability.

Viral derived overexpression of agouti in the LH, but not the PVN or DMH increased the sensitivity for diet induced obesity (Chapter 2). However, although the rats increased the intake of a high fat diet, this does not mean that they have an increased preference for fat. Future studies should be performed to investigate the role of local overexpression of agouti on food preference in a choice diet. Nevertheless, given the extensive reciprocal projections from the LH to the nucleus accumbens and amygdale (1), it could be argued that decreased MC signaling in the LH results in diet induced obesity because it alters food preference.

Although we did not investigate the involvement of local NPY overexpression in diet induced obesity or food preference, site specific effects can also be expected in this system. NPY$^{-/-}$ mice have a decreased intake of high fat and are therefore less sensitive to diet-induced obesity (243). This could be partly due to reduced NPY signaling in the DMH, since it has been shown that AAV-induced overexpression of NPY in the DMH results in an increase in high fat food intake and thereby diet-induced obesity (395). Acute NPY administration ICV or in the PVN is reported to preferentially increases high carbohydrate intake over high fat or high protein intake (221;396). In addition, ingestion of carbohydrates correlates positively with NPY expression levels in the PVN and DMH but not with expression in other hypothalamic areas (397). A similar correlation between fat or protein intake and hypothalamic NPY levels could not be observed (397). Based upon these data, it could be speculated that the relation between NPY signaling and carbohydrate intake may

reflect the need for fast energy sources in the beginning of the feeding cycle. Indeed, in the early dark period, both carbohydrate intake and NPY-induced effects on feeding are maximal (398;399). However, repeated injections of NPY in the PVN increase besides carbohydrate intake also fat intake (226). The NPY-induced increase in fat intake in a choice diet depends on the source of carbohydrates (400), but also on the basal preference of the animal (401). This shows the complexity of food preference and underlines the importance of aligning studies on macronutrient food intake.

Nevertheless, together, these data suggest that whereas the MC system can specifically alter the preference for high fat, the NPY system is also involved in the intake of carbohydrates. Furthermore, the brain areas where the food preferences are mediated differ per system. Fat preferences may relate via MC signaling in the LH to higher brain centers involved in the rewarding aspects of food, while carbohydrate preferences vary with NPY signaling in the PVN. Whether also other hypothalamic areas are involved in the regulation of macronutrient intake by the NPY or MC system could easily be further explored using AAV.

Circadian rhythms

Neuropeptides in the Arc show a diurnal expression pattern. These patterns are found for NPY, but also for AgRP and POMC, although data about the precise peaks and nadirs are not conclusive (402-405). The daily changes in neuropeptide expression probably relate to the nocturnal feeding rhythm of rats. However, not much is known about the pattern of MC secretion in projection areas of Arc. Although protein levels of NPY in the PVN seem to vary over the day, in other hypothalamic nuclei, including the perifornical lateral hypothalamus, no significant variation in NPY levels could be found (402;406). This suggests that the PVN rather than the LH is involved in an NPY induced feeding rhythm. Nevertheless, AAV-mediated overexpression of NPY in the LH resulted in a flattening of the circadian feeding and temperature rhythm, which was not observed after NPY overexpression in the PVN (Chapter 6).

Stimulation of the LH is known to initiate feeding even in satiated rats (365). Furthermore, the LH contains neurons that produce orexin, a neuropeptide that is involved in both food intake and wakefulness (29;407). NPY injections in the LH induce wakefulness, probably via orexin (372), and chronic administration of orexin disrupts circadian feeding patterns (407;408). Therefore, it could be argued that the sustained high levels of NPY in the LH after AAV-NPY injections continuously stimulate orexin neurons, thereby inducing a status where the rat is almost constantly awake and eating. This theory is strengthened by the fact that MCH and orexin neurons from the LH receive projections from the suprachiasmatic nucleus (SCN) (373), which drives the circadian feeding rhythm of feeding activity. Although we could not detect an increase in orexin mRNA in LH-NPY rats, it is

possible that orexin levels that are released are increased. Nevertheless, this remains to be investigated.

Conclusions

At first sight, increased NPY signaling and decreased MC signaling indeed have much similarities. However, when carefully analyzed, the systems can be dissociated. In addition, comparable effects are mediated by different nuclei (Figure 1). Although diet-induced obesity for example is related with NPY signaling in the DMH, MC signaling in the LH but not the DMH results in increased weight gain on a high fat diet (Chapter 2, (395)). Although we can not rule out that differences between viral-mediated increased NPY or reduced MC signaling are due to differences in levels of overexpression of NPY and agouti, there is evidence suggesting that they indeed have separate functions.
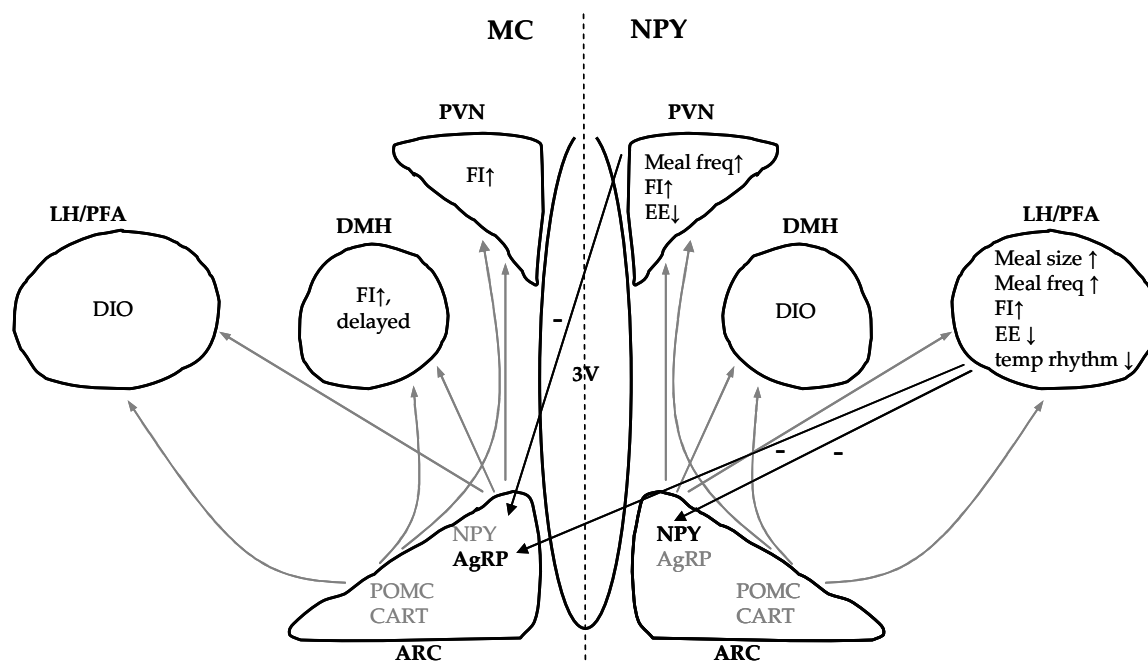
Figure 1: Overview of the effects of AgRP and NPY mediated by the different hypothalamic nuclei as expected by AAV-mediated local overexpression. DIO: diet induced obesity, FI: food intake, EE: energy expenditure.

While the NPY system is mainly involved in the drive to eat, the initial role of the MC system in food intake is inhibitory. The MC system is rather unique, in the sense that, besides by agonists, it is also regulated by endogenous antagonists, which have a stimulating effect on food intake. Considering the initial effects of both systems, it is therefore possible that while the melanocortin system overrules in situations when food is continuously available, the NPY system is the prominent system in situations when food is scarce. Together with a suppressed activity of the melanocortin system, mediated by AgRP,

this results in a rapid and large food intake whenever possible, combined with low energy expenditure. This could explain why NPY expression is not altered following AAV-agouti injections (chapter 2), since these animals do have continuous access to food. It would be interesting to investigate how neuropeptide expression in the Arc are changed when AAV injected animals are exposed to food restriction.

The PVN is a very important nucleus for both systems, immediately responding to an increase in NPY or a decrease in MC signaling with increasing food intake (Chapters 2 and 4). It remains to be established whether the MC system, as the NPY system, is also able to regulate energy expenditure via the PVN. In both the NPY and the MC system, the LH seems not to be implicated in food intake itself, but via NPY more in aspects leading to feeding and via the MC system more in diet induced obesity and possibly in food preference (Chapters 2 and 6). Also the DMH does not seem to have a direct role in food intake, since agouti is only able to increase food intake after three weeks of local overexpression (Chapter 2), and NPY signaling only results in increased food intake and body weight gain on a high fat diet (395). In view of the connections of the second order neurons in the LH with the SCN, and the lack of response to altered neuropeptide expression in the Arc, it is possible that the LH might not play a major role in feeding rhythms induced by arcuate NPY projections, as the PVN does, reflecting the peripheral need to feed. On the other hand, it seems that the LH, rather than the PVN, is involved in the feeding rhythm induced by the SCN, reflecting the day-night feeding schedule.

The MC system is, of course, besides by the inverse agonist AgRP also regulated by agonists. Future studies should be performed to determine whether increased MC signaling by chronic overexpression of MC agonists results in the exact opposite effects as chronic overexpression of AgRP or agouti. AAV-mediated overexpression of the multimeric α-MSH construct described in chapter 3 could be used to investigate this.

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NEDERLANDSE SAMENVATTING

Obesitas is een groeiend probleem in de westerse maatschappij. In de moderne levensstijl is (calorierijk) voedsel in overvloed aanwezig, en de noodzaak om te bewegen laag. In deze situatie gaan hogere hersengebieden, die bijvoorbeeld betrokken zijn bij het lekker vinden van bepaald voedsel, een steeds grotere rol spelen, waardoor verzadigings signalen genegeerd worden. Hierdoor wordt de homeostatische regulatie verstoord, met als gevolg een stijgend aantal gevallen met overgewicht. Het is daarom van groot belang om meer inzicht te krijgen in de regulatie van energiebalans.

De centrale regulatie van energie homeostasis omvat een complex neuraal circuit, opgebouwd uit verscheidene hersenkernen en neuropeptiden. Farmacologische en genetische studies hebben duidelijk gemaakt dat het melanocortine (MC) systeem en het neuropeptide Y (NPY) systeem een belangrijke rol spelen in deze regulatie van energiebalans (beschreven in hoofdstuk 1). NPY en agouti (een endogene antagonist van het MC systeem) zijn beide eetlustopwekkende neuropeptiden. Chronische toediening van deze peptiden in het brein leiden dan ook tot obesitas, een effect wat mede wordt toegeschreven aan veranderde signalering in de hypothalamus, een hersengebied dat een belangrijke rol speelt in de regulatie van voedselinname en lichaamsgewicht. Ondanks dat er al veel bekend is over deze neuropeptiderge systemen in de hypothalamus, is het nog niet precies bekend wat hun specifieke rol is in de verschillende hypothalame kernen.

Het onderzoek beschreven in dit proefschrift richt zich op de bijdrage van de verschillende gebieden in de hypothalamus van de rat in de door NPY en MC veroorzaakte effecten in de regulatie van energiebalans. Om de plaatselijke effecten van veranderde NPY- of MC signaaloverdracht te bestuderen is gebruik gemaakt van virale gen overdracht. Recombinante adeno-geassocieerde viruspartikels (AAV) kunnen, als een van de weinige virussoorten, ook niet-delende celtypes (zoals neuronen) infecteren en hebben als kenmerk dat het gen van interesse langdurig tot expressie gebracht wordt. Door deze AAV partkels coderend voor NPY of agouti in te spuiten op een van te voren berekende plaats in de hersenen kan een lokale overexpressie van agouti of NPY bewerkstelligd worden in hypothalame kernen waarvan al bekend is dat ze een belangrijke rol spelen in energiebalans.

Het melanocortine systeem bestaat uit agonisten (stoffen die de MC receptoren kunnen activeren) zoals α-MSH, en antagonisten (stoffen die de MC receptoren kunnen blokkeren) zoals AgRP en agouti. Het is bekend dat verminderde signaaltransductie via de MC4 receptoren, zowel door mutaties in de receptor zelf als door (algehele) overexpressie van agouti, leidt tot obesitas. Om meer duidelijkheid te krijgen welke hypothalame kernen een rol spelen in de ontwikkeling van obesitas bij verminderde signalering via MC4 receptoren zijn in hoofdstuk 2 de effecten van viraal-gemedieerde overexpressie van agouti in verschillende hypothalame kernen beschreven. Terwijl overexpressie van agouti in de

paraventriculaire nucleus (PVN) van de hypothalamus vrijwel direct resulteert in een verhoging van de voedselinname, met als gevolg een versnelde toename van lichaamsgewicht, zijn deze effecten na injectie van het AAV-agouti in de dorsomediale kern van de hypothalamus (DMH) pas te zien na drie weken. Overexpressie van agouti in de laterale hypothalamus (LH) daarentegen heeft geen enkel effect op de inname van voedsel en de toename van gewicht, behalve als de ratten een vetrijk dieet te eten krijgen.

Een andere benadering om meer inzicht te krijgen in de lokale rol van het MC systeem in de regulatie van energiebalans zou zijn om α-MSH tot overexpressie te brengen in de hypothalame kernen. Omdat α-MSH een vrij lage affiniteit voor de MC4 receptor heeft, zouden hoge concentraties van het peptide nodig zijn om effecten te bewerkstelligen. Omdat bekend is dat multimerisatie van peptiden de affiniteit voor receptoren kan verhogen, zijn in hoofdstuk 3 de bouw en farmacodynamische eigenschappen van multimeer α-MSH beschreven. De resultaten laten zien dat naast de affiniteit om aan de MC4 receptor te binden, ook de effectiviteit om deze receptor te activeren verbetert bij de multimerisatie van α-MSH. Toekomstig onderzoek zal moeten uitwijzen of AAV vectoren coderend voor dit multimere α-MSH ook in de rat in staat zijn om de MC4 receptor te activeren en zo meer duidelijkheid te geven in de lokale rol van het MC systeem in energiebalans.

De resultaten in hoofdstuk 4, 5 en 6 laten de effecten van NPY overexpressie in de PVN en LH op de ontwikkeling van obesitas zien. Hoofdstuk 4 beschrijft de effecten van een drie weken durende NPY overexpressie in de PVN. Naast een specifieke toename van eetgedrag in de lichtfase, veroorzaakt verhoogde NPY signalering in de PVN ook een afname in energieverbruik, gemeten in een verlaagde lichaamstemperatuur en activiteit van de ratten. Daarnaast wordt, nog voordat een effect op voedselinname duidelijk is, de secretie van insuline en leptine bevorderd. Samen resulteert dit in een zeer snelle ontwikkeling van obesitas.

In verdere studies is onderzocht wat de effecten van verhoogde NPY expressie op langere termijn zijn (hoofdstuk 5). In tegenstelling tot een verhoogde agouti expressie in de PVN, wat een blijvend effect heeft op voedselinname, heeft een overexpressie van NPY in de PVN maar een tijdelijk effect op eetgedrag. Vier weken na injectie van de virus partikels normaliseert de hoeveelheid geconsumeerd voedsel, evenals de (dagelijkse) toename in gewicht. Pair-fed studies (waarin de voedselinname van de met AAV-NPY geinjecteerde ratten gelimiteerd wordt tot die van de met controlevirus geinjecteerde ratten) wijzen uit dat de effecten afnemen wanneer een bepaald lichaamsgewicht bereikt is. Dit wijst erop dat NPY signalering in de PVN een rol speelt in de regulatie van een setpoint in gewicht.

In hoofdstuk 6 worden de effecten van vijftig dagen NPY overexpressie in de PVN en de LH vergeleken. AAV-NPY injecties in de LH resulteren in een vergelijkbaar fenotype als beschreven voor de PVN. Echter, de effecten op voedselinname zijn blijvend. Daar komt bij dat, terwijl een verhoogde NPY signalering in de PVN tot gevolg heeft dat alleen de

maaltijdfrequentie toeneemt (zonder dat de maaltijdgrootte beinvloed wordt), NPY overexpressie in de LH resulteert in zowel meer als grotere maaltijden.

Het gebruik van virale gen overdracht om de expressie van genen te veranderen is nog relatief nieuw. In hoofdstuk 7 is deze techniek vergeleken met de meer conventionele strategieen die gebruikt worden om meer inzicht te krijgen in de regulatie van energiebalans. Het grote voordeel van virale gen overdracht is dat injecties van adeno-geassocieerde virale (AAV) partikels in een volwassen rat resulteren in een stabiele, langdurige overexpressie van het gewenste neuropeptide. Hierdoor worden compenserende aanpassingen, die plaats kunnen vinden wanneer genexpressie wordt veranderd tijdens de ontwikkeling (in knockout en transgene modellen), gepasseerd. Ook kunnen de effecten van chronische veranderingen in concentraties van de neuropeptiden in een specifieke kern bestudeerd worden, wat niet mogelijk is bij infusie van de peptiden in de ventrikels (niet lokaal) of lokaal (niet chronisch).

In hoofdstuk 8 worden de resultaten van dit proefschrift samengevat. De beschreven resultaten geven aan dat NPY en agouti, ondanks de functionele overeenkomsten op het eerste gezicht, toch uit elkaar gehaald kunnen worden na een nauwlettende analyse. Daar komt bij dat de precieze functies van deze neuropeptiden afhangen van de hypothalame kern waarin ze tot expressie komen. In de PVN bijvoorbeeld is zowel het NPY als het MC systeem overduidelijk van belang voor de regulatie van voedselinname zelf, ookal verschilt de mate en duur van de geobserveerde effecten. De LH daarentegen lijkt meer een rol te spelen in aspecten die de voedselinname beinvloeden, zoals het 'lekker vinden' (via het MC systeem, zie hoofdstuk 2) of motorpatronen die voorafgaan aan eten (via het NPY systeem, zie hoofdstuk 6).

Tenslotte kan gezegd worden dat de virale benadering waarvoor gekozen is in dit proefschrift, inderdaad een goede methode is om de lokale rol van neuropeptiden in de hypothalamus te bestuderen. Verder onderzoek met behulp van virale vectoren kan, zeker in combinatie met de meer conventionele technieken, een beter inzicht geven in de regulatie van energiebalans, en daarmee hopelijk bijdragen aan strategieen om het aantal individuen met obesitas te verminderen.

CURRICULUM VITAE

Birgitte Tiesjema (Gitte) werd geboren op 11 februari 1978 te Bilthoven. In 1997 behaalde zij het VWO diploma aan het Montessori Lyceum Herman Jordan te Zeist, waarna ze datzelfde jaar begon aan de opleiding Medische Biologie aan de Universiteit Utrecht. Tijdens deze studie werden twee onderzoeksstages verricht. De eerste stage werd uitgevoerd bij het Jordan Laboratorium voor Hemato-Oncologie van de afdeling Hematologie, in het Universitair Medisch Centrum Utrecht. Onder begeleiding van Dr. A.C.M. Martens en Dr. H. Rozemuller heeft zij gewerkt aan de constructie van groeifactor toxine fusie-eiwitten voor de behandeling van leukemie. De tweede stage werd uitgevoerd bij de afdeling Farmacologie en Anatomie van het Rudolf Magnus Instituut voor Neurowetenschappen, waar de lange termijn effecten van neonatale glucocorticoid behandelingen bij de rat bestudeerd werden. Deze stage werd uitgevoerd onder begeleiding van Prof. Dr. V.M. Wiegant en Dr. P.J.G.H. Kamphuis, in samenwerking met Dr. P.J. Lucassen van het Swammerdam Institute for Life Sciences van de Universiteit van Amsterdam. In 2002 werd de doctoraal opleiding met goed gevolg afgesloten en werd zij aangesteld als assistent in opleiding bij de afdeling Farmacologie en Anatomie van het Rudolf Magnus Instituut voor Neurowetenschappen onder begeleiding van Prof. Dr. R.A.H. Adan en Dr. S.E. la Fleur. De resultaten van het onderzoek naar de moleculaire mechanismen die ten grondslag liggen aan de centrale regulatie van energiebalans staan beschreven in dit proefschrift.

LIST OF PUBLICATIONS

Kas MJH, **Tiesjema B**, van Dijk G, Garner KM, Barsh GS, ter Brake O, Verhaagen J, Adan RAH. (2004) Induction of brain-region specific forms of obesity by Agouti. *J Neurosci* 24:10176-10181.

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Tiesjema B, la Fleur SE, Luijendijk MCM, Brans MAD, Lin ED, During MJ, Adan RAH. Injection of rAAV-NPY in the paraventricular nucleus results in obesity. *Submitted*

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Tiesjema B, la Fleur SE, Luijendijk MCM, Kalsbeek A, Adan RAH. Differential effects of rAAV-mediated NPY overexpression in the PVN and LH on energy balance. *In preparation*

Tiesjema B, Merkestein M, Garner, de Krom M, Adan RAH. Multimeric α-MSH has increased efficacy to activate the MC4 receptor. *In preparation*

Tiesjema B, Heine VM, Kamphuis PJGH, van Bel F, Wiegant VM, Lucassen PJ. Neonatal dexamethasone (DEX) treatment affects neurogenesis in the adult rat hippocampus. *In preparation*

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