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.

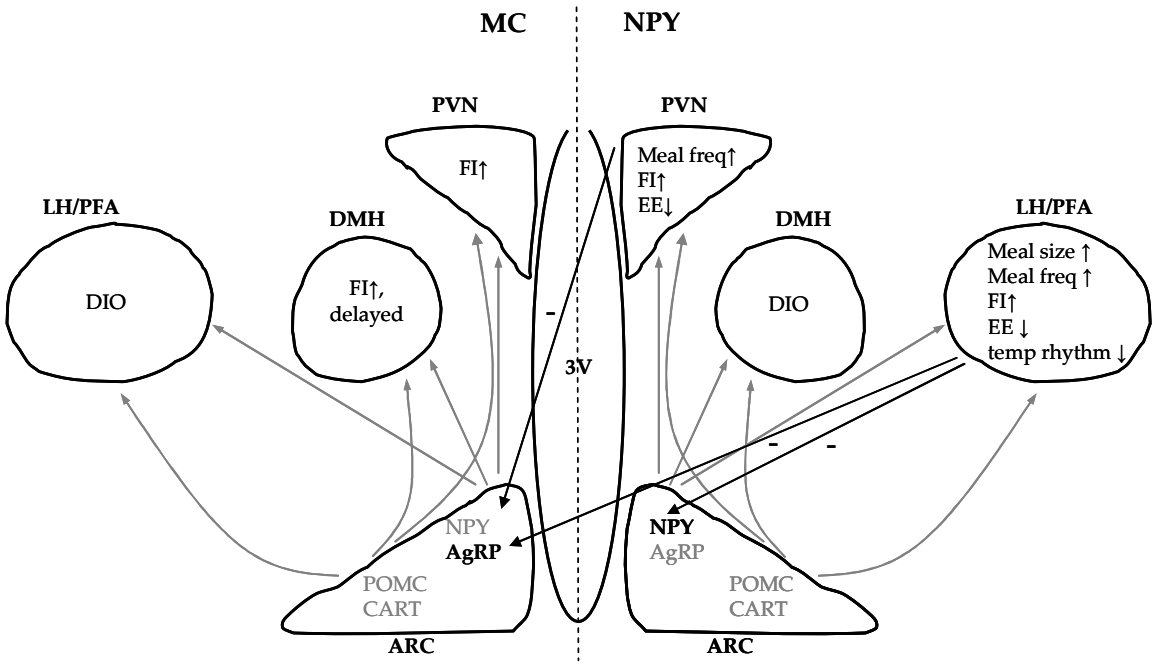

Figure 1: Overview of the effects of AgRP and NPY mediated by the different hypothalamic nuclei as expected by AAV-mediated local overexpression. DIO: diet induced obesity, FI: food intake, EE: energy expenditure.

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.