Brain circuitries in control of feeding behaviors

Focus on Neuropeptide Y

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Chapter XI.
Summary and General Discussion

The high prevalence of obesity, with a key role of the human-engineered obesogenic dietary environment in driving its development and maintenance, warrants a better understanding of the processes underlying food intake regulation as well as the dysregulation by palatable dietary components, such as highly saturated fats and simple sugars. In this thesis, diet-induced obesity was modelled by giving male Wistar rats unlimited access to a free-choice high-fat high-sugar diet (fcHFHS), consisting of four diet components, namely chow (high in complex carbohydrates), a bottle of water, a bottle of sucrose solution, and a dish of saturated fat. This diet model has a high clinical validity to model human diet-induced obesity as it provides dietary variety through choice options, and reliably leads to sustained hyperphagia and metabolic changes that are similar to those during human obesity (Slomp et al., 2019).

The brain is central to the regulation of energy balance by aligning energy uptake (via caloric intake) and energy expenditure (e.g. via metabolism or physical activity). The neural circuits related to caloric intake regulation are often categorized into homeostatic- and reward circuitries. The homeostatic circuitries consist of brain regions in the hypothalamus and hindbrain, which are located in close proximity to the more permeable parts of the blood-brain barrier, thus allowing easy access of blood-borne signals, which reflect the energy status of the animal. These areas regulate homeostasis in energy balance. The principal reward-related circuitry consists of the mesolimbic dopamine system that projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), as well as to other corticolimbic structures. The reward-related circuitries lie deeper in the brain and regulate the hedonic aspects of (palatable) food intake as well as the motivation to obtain food. The peptide Neuropeptide Y (NPY) plays a role in both circuitries by increasing both caloric intake as well as the motivation to obtain food.

In this thesis, we investigated the effects of diet composition and energy status on NPY function in several brain regions that are important for the homeostatic and/or hedonic control of food intake. We also studied the role of NPY in dietary selection by reviewing the literature on this topic and animal experimentation. In addition, we also investigated the anatomical organization of NPY in the reward-related circuitry. Lastly, we investigated how changes in the diet can result in adaptations in the reward-related circuitry, for example, after a short fast or after sustained hyperphagia associated with our fcHFHS diet model of obesity.

Summary of the main findings

Dietary effects on Npy gene expression in homeostasis- and reward-related brain regions

Chapter II provides an overview of how consumption of different diets can lead to changes in the NPY peptide- and *Npy* gene expression levels in homeostasis- and reward-related brain regions. Consumption of carbohydrate-rich diets most often results in higher NPY levels in

two regions of the hypothalamus, the arcuate nucleus (Arc) and the paraventricular nucleus (PVN), while consumption of fat-rich diets results in lower NPY levels in these regions. The Arc contains NPY neurons that are sensitive to blood-borne hormones and metabolites, and convey these signals to several regions, including the PVN, via neuronal projections. The Arc->PVN projection is very important in mediating the effects of NPY on food intake. Animals that had consumed diets that were rich in fat and sugar showed increased *Npy* expression in the Arc one week after the start of the diet, suggesting that they would they would remain hyperphagic. After four weeks of fcHFHS diet consumption, Arc *Npy* expression was normalized in these animals, but instead, they were more sensitive to intracerebral NPY infusions. Our literature study also showed that the effects of diet on NPY levels had not yet been measured in reward regions in animals consuming a fcHFHS diet. Also, changes in NPY receptor levels (i.e. NPY1R, NPY2R, NPY4R, and NPY5R) could provide a mechanism for increased sensitivity to NPY in rats on a free-choice diet. However, this hypothesis had not been addressed comprehensively. In **Chapter III**, we therefore measured *Npy*, *Npy1r*, *Npy2r*, *Npy4r*, and *Npy5r* gene expression after six weeks of fcHFHS diet consumption in four brain regions; the Arc, as a control, the lateral hypothalamic area (LHA), because intra-LHA NPY infusion elicits one of the largest hyperphagic responses, and in two of the most important reward-related brain regions, the VTA and NAc. Consumption of the fcHFHS diet altered gene expression in the LHA and NAc. Indeed, *Npy* expression was increased in the LHA, whereas *Npy1r* expression was differently regulated during the day in the NAc compared to chow-fed controls. These findings suggest that diet-induced changes in NPY sensitivity should be investigated at the peptide level for receptors. In addition, these findings suggest a role for NPY neurons in the LHA in mediating the effects of diet-induced changes in food intake regulation.

Our literature study, showed that, at the time, very little was published on the regulation of NPY signaling in the LHA as well as in the reward-related brain regions. The Arc NPY population is highly sensitive to changes in energy status, such as fasting, which increases *Npy* mRNA expression in the Arc. However, it was unknown if *Npy* expression in the LHA, VTA, and NAc were similarly affected by a negative energy status. Therefore, in **chapter IV**, we investigated if a 24-hour fast could alter *Npy* expression in the LHA, VTA and NAc, as well as the effects of fasting on *Npyr* expression. Our study shows that a 24-hour fast indeed increased Arc *Npy* levels, and that Arc *Npy1r* levels decrease due to fasting when measured at the beginning of the light period. In the dark period, only a fasting-induced decrease in Arc *Npy2r* expression was observed. Fasting did not affect *Npy* or *Npyr* levels in the LHA, or in either of the reward-related brain regions. This suggests that NPY has a different role in the Arc than in the LHA, or the reward-related brain regions. In addition, these findings indicated that the NPY1R and NPY2R subtypes play a role in the adaptive changes during fasting.

The effects of NPY infusion on dietary choice depends on brain region and dietary composition

A previous study from our research group showed that intraventricular infusion of NPY increased intake of chow and fat in rats on the fcHFHS diet. The literature indicated that the regulation of dietary choice by NPY could be different depending on brain region. For example, the NAc was reported to play an important role in the regulation of fat intake. In **Chapter V**, we show that the effect of NPY on fat intake is mediated by NPY signaling in the NAc in rats consuming the fcHFHS diet. In this study, we also show that NPY1R signaling in the NAc mediated these effects, as prior NPY1R antagonist infusion in the NAc blocked the effects of NPY on fat intake. Subsequently, we show in **chapter VI**, that NPY signaling in the LHA could contribute to the increase in chow intake after intraventricular NPY infusion in fcHFHS-fed rats. NPY in the LHA leads to one of the greatest hyperphagic responses compared to several brain regions. In addition, we show several diet-induced changes in the LHA NPY system in fcHFHS-fed rats compared to CHOW-fed control rats. In rats consuming a chow-diet, intra-LHA NPY infusion can be blocked by prior infusion of an NPY1R or NPY5R antagonist, whereas only blocking NPY5R, and not NPY1R, is effective to block intra-LHA NPY-induced feeding in fcHFHS-fed rats.

NPY can also increase food intake when administered into the PVN, one of the brain regions that is important for the homeostatic control of energy balance. The orexigenic effects of NPY in the PVN depend on the composition of the basal diet of the animals. For instance, if rats consume high levels of carbohydrates, intra-PVN infusion increases the intake of carbohydrates specifically. If rats consume high levels of fat, intra-PVN infusion additionally increases the intake of fat. It was, however, unknown if the orexigenic effects of NPY in the LHA are also dependent on the composition of the basal diet, which we investigated in **chapter VII**. Rats could consume either the standard chow diet, the fcHFHS diet, or a free-choice diet with access to only one of the palatable food items; a free-choice high-fat (fcHF), or a free-choice high-sucrose (fcHS) diet. We show that the effects of intra-LHA NPY on food intake were also dependent on the composition of the basal diet. Rats that consumed the standard diet, the fcHFHS diet, or the fcHF diet specifically increased the intake chow (mainly complex carbohydrates). In the fcHFHS group, two groups could be distinguished that consumed either a high fat::sucrose ratio (relatively high intake of fat and a low intake of sucrose water; fcHFHS-high fat group [fcHFHS-hf]) or a low fat::sucrose ratio (relatively low intake of fat and a high intake of sucrose water; fcHFHS-low fat group [fcHFHS-lf]). In the fcHFHS-hf group, intra-LHA NPY also increased intake of both the chow and fat components. Interestingly, rats consuming a fcHS diet were insensitive to intra-NPY infusion. Future studies will have to determine if these diet-induced changes in LHA NPY responsivity are adaptive or maladaptive.

The origin of NPY in reward brain regions

NPY is expressed in projecting neurons and interneurons throughout the brain. Several areas receive NPY projections and show dense innervating fibers. Historically, NPY has been studied primarily in the homeostatic brain regions with particular attention for the Arc->PVN projection in the regulation of food intake. However, the Arc NPY neurons also project to the LHA, and the C1/A1 area of the hindbrain also sends NPY projections to the PVN. The origin of NPY in the reward areas is however less well studied. The NAc contains neurons that express NPY, but it was unknown if NPY signaling in the NAc also originated from afferent projections. **Chapter V** therefore used retrograde tracing to determine that Arc NPY neurons also project to the NAc. For the VTA, a consensus on the presence of NPY-expressing neurons in the VTA had not yet been reached. In **chapter VIII**, we systematically investigated the origin of NPY in the VTA and concluded that the VTA does not contain NPY/*Npy*-expressing neurons under normal physiological circumstances in male rats. In addition, retrograde tracing showed that NPY neurons in the Arc and C1/A1 area in the hindbrain project to the VTA. The regions of the reward system can thus both receive information from homeostatic regions that can sense signals related to energy status.

Effects of energy status on gene expression in the reward circuitries

In addition to the hypothalamus, and specifically the NPY system, it has also been shown that the reward circuitry is sensitive to fasting. The dopamine and opioid system regulate different aspects of reward; the dopamine system regulates motivational aspects ('wanting'), whereas the opioid system regulates the rewarding aspects ('liking'). Fasting increases the motivation to obtain food, and may also increase the hedonic impact of food. In addition, the dopamine system is known to be responsive to energy balance-regulating hormones that signal via G-protein coupled receptors, which suggests that changes in gene expression may be involved in mediating the effects on the dopamine system. In **Chapter IX**, we investigated if a short period of fasting can modulate gene expression in the dopamine system or the opioid system. Fasting primarily affected gene expression in the dopamine reward system, and primarily in the VTA, the origin of mesolimbic dopamine neurons. Gene expression for the dopamine D2 receptor and tyrosine hydroxylase (the rate-limiting enzyme for the synthesis of dopamine) were decreased after fasting at a time when rats would normally prepare for feeding. These findings were generally in accordance with the effects of chronic food deprivation on the dopamine system, and show that energy status can affect the dopamine system, possibly to modulate food-motivation.

Effects of dietary composition on the dopamine system in the reward system

The reward system is different between people with obesity and lean controls. For example, obese people have lower dopamine D2/3 receptor ($DRD_{2/3}$) binding in the striatum, of which

the NAc is a subregion. Previous studies in our research group had demonstrated that dietary composition modulates the $DRD_{2/3}$ binding in the NAc. Basal fat intake was linked to changes in the $DRD_{2/3}$ binding in the striatum and to the response of rats to an amphetamine injection. However, to measure $DRD_{2/3}$ binding with storage phosphor-imaging, the animal has to be sacrificed. To study how the effects of diet on striatal $DRD_{2/3}$ receptors evolve over time, a method is required to determine $DRD_{2/3}$ availability in a non-invasive way and without sacrificing the animal. In **chapter X**, we investigated if the response to an amphetamine injection could predict the availability of $DRD_{2/3}$ in the different subregions of the striatum. In rats consuming a standard chow diet, the response to an amphetamine injection could predict the amount of dorsal striatal $DRD_{2/3}$ binding. However, in the rats consuming the fcHFHS diet, the relationship between amphetamine response and $DRD_{2/3}$ binding was abolished.

General Discussion

The regional NPY systems in the brain are differentially affected by energy status

We investigated the NPY systems in several brain regions and how they are affected by changes in energy status such as overconsumption (fcHFHS model; chapter III) and fasting (24 hour-fasting period; chapter IV). Our findings indicate that changes in energy status can affect the NPY systems of the Arc, LHA, and NAc differentially; the Arc responds to both overconsumption and fasting, whereas the LHA and NAc NPY systems respond mostly to overconsumption. Interestingly, we find that the NPY system in the VTA does not respond to changes in energy status.

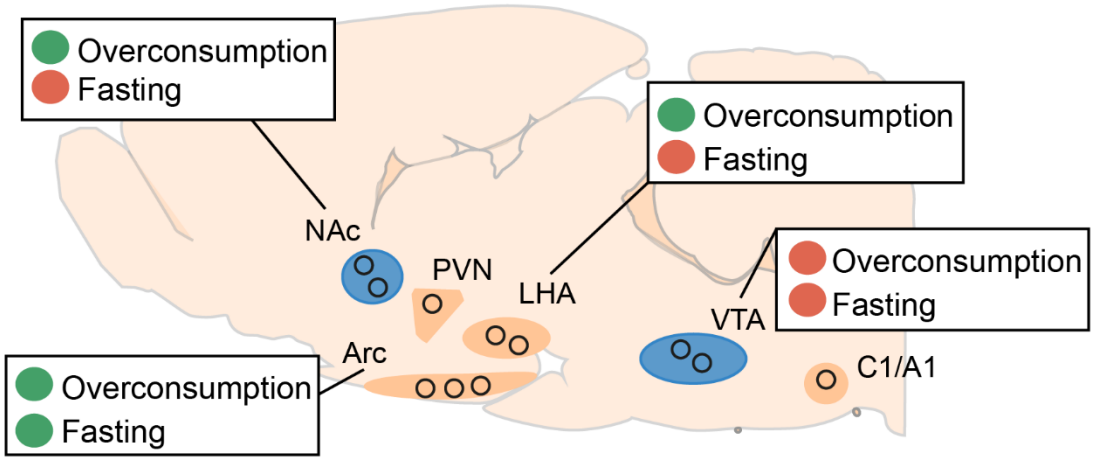

Figure 1. Brain region-specific sensitivity to changes in energy status. The Arc NPY system is sensitive to positive and negative changes in energy balance, whereas the NAc and LHA NPY systems are mostly sensitive to overconsumption, and the VTA NPY system to neither (chapter III, IV). The effects of overconsumption were measured after six weeks *ad libitum* access to the free-choice high-fat high-sugar diet, and the effects of fasting after a 24-hour fasting period. Green = NPY-related gene expression is affected by the manipulation, Red = NPY-related gene expression is not affected by the manipulation, Orange areas = areas of the homeostatic system, Blue areas = areas of the reward system. See text for details.

The Arc NPY neurons lie close to the median eminence and partial blood-brain-barrier, and are sensitive to both positive and negative changes in energy status ([Kohno & Yada, 2012; Rodriguez et al., 2010]; chapters II, III). Our findings indicate that the Arc responds to rapid changes in energy status, for instance after short term overconsumption (chapter II; [la Fleur et al., 2010]) or an acute fast (chapter IV), but the effects on *Npy*/*Npyr* gene expression are normalized after long term overconsumption (chapter IV; [van den Heuvel, Eggels, van Rozen, et al., 2014]) or chronic food restriction (Bi et al., 2003). Future studies will have to examine

whether, and to what extent, the Arc NPY neurons are capable of dynamically sensing perturbations of energy status after normalization of *Npy* mRNA levels. The Arc->PVN axis is predominantly researched in relation to changes in energy status and is known to regulate feeding behavior, and has therefore been omitted in the studies included in this thesis. In general, NPY output in the PVN mimics Arc NPY levels and fluctuate with fasting and re-feeding ([S. P. Kalra et al., 1991; Sahu et al., 1988], but not always [Beck, Jhanwar-Uniyal, et al., 1990]). In addition, the NPY neurons in the C1/A1 area of the hindbrain can respond to a low energy status that mimics fasting (A. J. Li & Ritter, 2004). It is, however, unknown if the C1/A1 NPY neurons respond to overconsumption.

The NPY systems of the LHA and NAc were, however, not affected by acute fasting, but were affected by overconsumption; i.e. both regions show altered expression of NPY-related genes during fcHFHS overconsumption (chapters III, IV). It has been shown that NPY peptide levels in the LHA are reactive to re-feeding after a fast, but not by fasting itself (Beck, Jhanwar-Uniyal, et al., 1990), and LHA NPY/*Npy* levels are affected during overconsumption of an obesogenic diet (chapter II; [Beck, Stricker-Krongrad, et al., 1990; Beck, Stricker-Krongrad, Burlet, et al., 1992; Wilding, Gilbey, Jones, et al., 1992; Wilding, Gilbey, Mannan, et al., 1992]). For the NAc, very few studies have looked at the NPY system and its responsiveness to energy status. We found that *Npyr* gene expression is differentially modulated by fcHFHS diet consumption (chapters III). Though a previous study showed that NAc NPY peptide levels were not altered by consumption of an obesogenic diet in very young rats (Beck et al., 1994), this has not been measured in adult rats to date. To confirm that the NPY systems of the LHA and NAc are primarily involved in mediating overconsumption, future studies should determine the reactivity of the LHA- and particularly the NAc NPY systems to different physiological perturbations of energy status.

It was surprising that the NPY system of the VTA was not responsive to a positive or negative change in energy status (chapters III, IV), as studies have increasingly shown that the VTA is responsive to blood-borne signals that convey energy status. Also, it is clear that a change in energy status can affect NPY/*Npy* levels in the Arc (Kohno & Yada, 2012; Skibicka, Hansson, Alvarez-Crespo, Friberg, & Dickson, 2011), and we found that energy status-responsive NPY neurons in the Arc and C1/A1 region in the brainstem project towards the VTA (chapter VIII; [Hahn et al., 1998; A. J. Li & Ritter, 2004]), suggesting that energy status-induced changes in NPY output are able to affect *Npyr* expression in the VTA through this connection. However, both long-term overconsumption or fasting did not affect gene expression of the NPY system in the VTA (chapters III, IV). We do, however, believe that our manipulations of energy status should be sufficient to elicit changes in the VTA NPY system if it was responsive to these factors. Indeed, our manipulations of energy status elicit changes in *Npy* gene expression in other brain regions, such as the Arc and LHA (chapters III, IV), and behaviorally, different effects have been reported to occur that are linked to VTA function.

Both long-term overconsumption and fasting can increase the motivation to obtain food (Jewett et al., 1995; la Fleur et al., 2007). Moreover, fasting can affect dopamine neurotransmission in the VTA (Roseberry, 2015), and we show that fasting can alter dopamine gene expression in the VTA (chapter IX).

In accordance with our findings, previous studies had shown that two weeks of overconsumption did not increase VTA NPY peptide levels (table 3 chapter II; [Beck, Stricker-Krongrad, et al., 1990; Beck, Stricker-Krongrad, Burlet, et al., 1992]). However, it is important to note that Arc NPY levels were not consistently increased in the models of overconsumption employed in these studies. In addition, our measurements after overconsumption are also at a time point when Arc *Npy* levels are normalized. Alternatively, it could be that changes in the VTA NPYRs are present at the protein level instead of at the gene expression level. Future studies could investigate this.

Based on our observations, the Arc is indicated as the primary site where energy status is sensed to dynamically alter the local NPY system. For the PVN it is clear that it receives and processes (a derivative of) this information. Neurons in the LHA, NAc and VTA can sense energy status, however, the NPY systems at these sites are not always involved in sensing energy status. In the LHA and NAc, the NPY system is responds to positive changes in energy status, whereas the NPY system in the VTA does not respond to either positive or negative changes in energy status. Future studies measuring NPY and NPYRs at the protein levels in these regions during dynamic changes in energy status are still necessary to resolve several outstanding questions.

A distributed and interconnected NPY system regulates different aspects of food intake

In this thesis, we determined the effects of NPY in the NAc and LHA on food intake. Our data show that activation of the NPY1R in the NAc can elicit intake of the fat component in rats that consume a fcHFHS diet (chapter V), whereas NPY in the LHA increases intake of chow, or chow and fat depending on prior dietary preferences in fcHFHS diet-fed rats (chapter VII). Together with the roles of the Arc, PVN, VTA and C1/A1 NPY neurons in food intake regulation, and the neuroanatomical connections of the NPY neurons, the findings in this thesis indicate that a distributed and interconnected NPY system regulates different aspects of feeding behavior depending on brain region (see Figure 2).

<u>NPY induces different feeding-related behaviors in the LHA and NAc</u>

Activation of Arc NPY neurons can increase food intake and food-motivation, NPY in the PVN can increase food intake, and in the VTA, it can increase food-motivation (Krashes et al., 2013; Pandit et al., 2014a; Stanley & Leibowitz, 1984). In this thesis, we focused on the NPY systems

of the LHA and NAc. Behavioral analysis after LHA and NAc NPY/NPYR-antagonist infusions indicated that these regions modulate food intake in a manner that is compatible with their roles in the homeostatic and reward systems.

The LHA is recognized as a transition region between homeostatic and reward circuitries due to its neuroanatomical interconnections (Morton et al., 2006). We, and others, show that NPY signaling in the LHA increases food intake of bland and palatable food items depending on prior dietary intake ([Stanley et al., 1993]; chapters VI, VII). In addition, intra-LHA NPY can also lead to increases in reward-related behaviors, though not consistently. For example, intra-LHA NPY can induce a conditioned place preference, but does not always increase the motivation to obtain sucrose pellets (C. M. Brown et al., 2000; C. M. Brown et al., 1998; Pandit et al., 2014a).

In addition, in the NAc, NPY increases intake specifically of a palatable food component, but does not increase intake when relatively unpalatable chow is provided (chapter V; [C. M. Brown et al., 2000; Pandit et al., 2014a]). Furthermore, NPY in the NAc can increase the motivation to obtain a sucrose pellet and other reward-related behaviors. Accordingly, intra-NAc NPY infusion can increase dopamine output (Sorensen et al., 2009), and the effects of intra-NAc NPY on food intake are reminiscent of the effects of NAc opioid signaling on fat intake (M. Zhang et al., 1998). The effects of NPY in the NAc are thus compatible with a reward system function.

We also show that the NPY1R and NPY5R may have a redundant role in the LHA for increasing food intake (chapter VI), and that the pharmacology of the NPY1R can change during one week overconsumption of fat and sucrose (chapter VI). In addition, consumption of a fcHS diet makes the LHA insensitive to NPY (chapter VII). Together this shows that the LHA NPY system is sensitive to dietary composition. Moreover, we have shown that the NPY1R underlies the effects of increased consumption of fat after intra-NAc NPY in rats consuming a fcHFHS diet for one week (chapter V). We did not investigate if dietary composition can affect NPYR signaling in the NAc by determining the effects of intra-NAc NPY on food intake in rats consuming different free-choice diets, or if other NPYR, such as the NPY5R, can also mediate changes in food-intake related behaviors. It is, however, possible that other NPYR in the NAc may play a role in increasing fat intake. Indeed, *Npy1r* and *Npy2r* gene expression are altered after six weeks fcHFHS diet consumption (chapter II), and *Npy5r* may be modulated by fasting (chapter III), suggesting that they are sensitive to energy status. In addition, as the changes in *Npyr* expression were seen after six weeks fcHFHS diet consumption, it is possible that changes in the NPYR responsivity, in particular the NPY1R, were not yet apparent in our study as rats were tested after one week of fcHFHS diet consumption. Future studies should assess the functionality of the NPYRs in the NAc after long-term fcHFHS diet consumption as well as the modulation of NPYR functionality by consumption of other diets such as the fcHS and fcHF diet. In addition, it is not clear whether

the changes in LHA NPY signaling are adaptive or maladaptive, which will need to be determined.

Together, the NPY systems of the different regions mediate different aspects of feeding behaviors, and the conceptualization of homeostasis- and reward-related behaviors broadly captures the function of NPY signaling in each system.

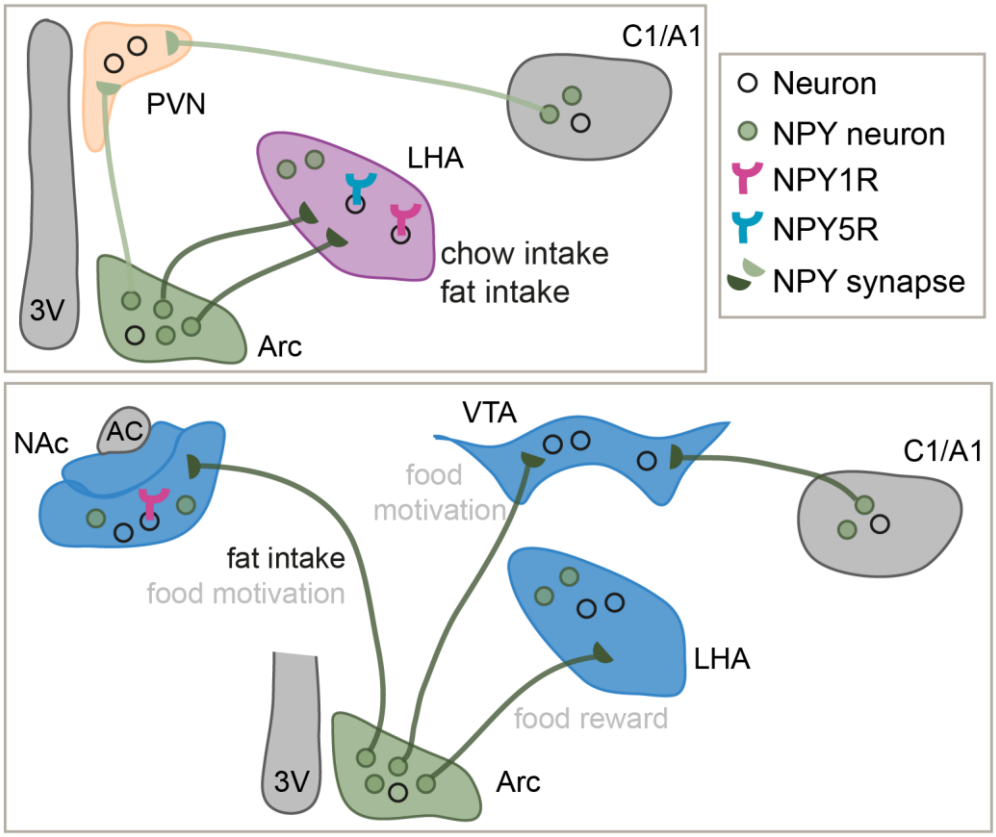

Figure 2. Overview of the brain region-specific functions of NPY in food intake regulation. Arc NPY neurons project to the PVN, LHA, NAc and VTA to regulate feeding behaviors. **Upper panel)** *NPY function in homeostatic brain regions*: Activation of Arc NPY neurons leads to increased food intake. The afferent NPY projections to the PVN modulate feeding behavior (not studied in this thesis). In the LHA, NPY signals via NPY1R and NPY5R to increase chow intake, and increases fat intake depending on prior dietary consumption. **Lower panel)** *Npy function in reward-related brain regions*: Arc activation also increases the motivation to obtain food, which could be via signaling in the VTA and/or NAc. In addition, the Arc->NAc projection may be involved in modulating feeding behavior by specifically increasing fat intake via the NPY1R. NPY signaling in the LHA may be involved in mediating food reward-related behaviors (not studied in this thesis). The role of the C1/A1 NPY projections and of the local LHA- and NAc NPY neurons in food intake regulation require further investigation. In addition, the role of the NPY5R in the NAc in feeding behaviors should also be investigated. 3V = third ventricle, AC = anterior commissure, Arc = arcuate nucleus, C1/A1 = catecholaminergic cell groups in the ventrolateral medulla of the brainstem, LHA = lateral hypothalamic area, NAc = nucleus accumbens, PVN = paraventricular nucleus, VTA = ventral tegmental area. Light-green projections and light-grey behaviors were not studied in this thesis. Only brain regions and NPY receptors that were studied in this thesis are included in the figure.

The Arc NPY projections as central regulator of food-related behaviors?

The Arc NPY neurons can sense positive and negative fluctuations in energy balance (see section: *The regional NPY systems in the brain are differentially affected by energy status systems*). Also, these neurons project to all downstream regions that affect food intake or food-motivated behaviors ([Bai et al., 1985; Elias et al., 1998; D. Wang et al., 2015]; chapters V, VIII). The Arc NPY neurons are thus well situated to control feeding-behaviors through downstream regions. Accordingly, activation of the Arc NPY neurons leads to increased food intake as well as increased food-motivation (Krashes et al., 2013).

The PVN and VTA do not harbor local NPY-expressing neurons. It was already shown that the Arc NPY neurons project to the PVN (Bai et al., 1985; D. Wang et al., 2015), and we show here that the Arc NPY neurons project to the VTA (chapter VIII). Though the C1/A1 NPY neurons also project to both regions ([Sawchenko et al., 1985]; chapter VIII), these neurons lie further away from a circumventricular organ that could provide rapid information on energy status (Paxinos & Watson, 2007). Therefore, it is likely that the Arc NPY neurons play a predominant role in affecting neurotransmission in the PVN and VTA to modulate feeding-related behaviors.

The LHA and NAc, however, do harbor neurons that express NPY, therefore the origin of NPY may also be local for these regions (chapter II, VII; [Broberger, Johansen, et al., 1998; Elias et al., 1998]). Unfortunately, both local populations have not been characterized with respect to their role in feeding behaviors. Based on previous research and the findings in this thesis, we hypothesize that the local LHA NPY neurons play an important role in mediating the effects of NPY on food intake. For instance, we have found that *Npy* expression is modulated by six weeks fcHFHS consumption (chapter II). Furthermore, when LHA NPY peptide levels decreased with refeeding after fasting, Arc NPY peptide levels were not modulated in a manner that could explain changes in LHA NPY output (Beck, Jhanwar-Uniyal, et al., 1990), making it most likely that the NPY changes occur in NPY interneurons located in the LHA. We localized *Npy*- and NPY-immunoreactive neurons to the posterior part of the LHA (chapters II, chapter VIII). Selectively targeting the LHA NPY neurons will aid elucidating their role in food intake regulation.

The role of local NPY neurons in the NAc in modulating feeding behaviors has not been examined. We show that NAc *Npy* mRNA is not sensitive to changes in energy status (chapters III, IV). As stimulation of Arc NPY neurons can increase food intake and motivation, and intra-NAc NPY can also stimulate both feeding and food-motivation (Krashes et al., 2013; Pandit et al., 2014a), it is thus likely that Arc NPY modulates food-related behaviors in the Nac. However, future studies will have to confirm this.

To ascertain the contribution of the Arc NPY neurons to food intake regulation in all downstream areas, we suggest specific Arc NPY neuron activation and simultaneous NPY protein analysis in the downstream areas. In addition, future studies could investigate the

relative contributions of the different NPY sources (i.e. the Arc, C1/A1 and local NPY neurons) in modulating feeding-related behaviors.

Neuropeptide Y-reward system interactions in the modulation of food-motivation

One of the initial goals of this thesis was to investigate NPY-dopamine interactions to determine if increased NPY levels could explain the increases food-motivation in rats consuming a fcHFHS diet (la Fleur et al., 2007). In this thesis, we show that the Arc NPY neurons project towards the NAc and VTA, providing a neuroanatomical basis for our hypothesis that increased Arc NPY levels are signaled to the reward system in diet-induced obesity (chapter VIII).

The role of the NPY-dopamine interaction in modulating food-motivation has been suggested because the increases in food-motivation after intra-NAc or intra-VTA NPY infusion can be reduced by blocking dopamine receptors (C. M. Brown et al., 2000; Josselyn & Beninger, 1993; Pandit et al., 2014a). Accordingly, NPY can increase dopamine output in the NAc and infusion of NPY5R agonists can mimic this effect (Quarta et al., 2011; Sorensen et al., 2009). In addition, we have shown that NPY1R activation leads to increased intake of palatable food, similar to the effects of NAc opioid signaling on fat intake (M. Zhang et al., 1998), and that NPY1R is expressed on opioid-peptide expressing neurons in the NAc, which harbor dopamine receptors that receive input from the VTA (chapter V). It thus seems probable that NPYR signaling can differentially affect dopamine- and opioid signaling to mediate the different effects of NPY on food-related behaviors. Future studies can thus investigate the relative contributions of dopamine and opioid signaling in mediating the effects of intra-NAc NPY on food intake. In addition, future studies can investigate if the roles of the NPY1R and NPY5R are exclusive to opioid signaling/palatable food intake or dopamine/motivational processes respectively, or if they can contribute to both.

Interestingly, in the VTA, NPY paradoxically inhibits dopamine neurotransmission (Korotkova et al., 2006; Sorensen et al., 2009; K. S. West & Roseberry, 2017). Based on earlier findings (Pandit et al., 2014a), and our own that VTA NPY gene expression does not respond to changes in energy status (chapters III, IV), we suggest that NPY-signaling in the VTA system might be involved primarily with mediating food-reward related behaviors, whereas the dopamine system can also respond directly to energy deficit. We show that fasting affects dopamine gene expression in the VTA only when measured just prior to the dark period (chapter IX), whereas the effects of fasting are measured only just after the onset of the light period in the Arc NPY system, and fasting does not affect the NPY system of the VTA at all (chapter IV). This suggests a dichotomy in the regulation of these systems during fasting. In addition, our findings indicate that signals of energy status can affect dopamine signaling in the VTA, but not NPY in the VTA ([Figlewicz et al., 2003]; chapter IV). Still, activation of Arc

NPY neurons also leads to increased motivation to obtain food, therefore a functional subpopulation of Arc NPY neurons may signal to the VTA to modulate food-motivation (Krashes et al., 2013; Marks et al., 1992). Future studies are required to elucidate the nature of NPY-dopamine interactions.

Future studies will have to directly measure the effect of intra-VTA NPY infusion on dopamine release in the NAc to determine how NPY-signaling can modulate the output of the dopamine system. In addition, future studies employing projection-specific NPY neuron activation and simultaneous behavioral analysis or NAc/VTA NPY peptide release measurements are necessary to elucidate the regulation of the NPY system in the NAc and VTA. Lastly, future studies will have to determine if NPY release is indeed increased in the NAc or VTA of rats that consume a fcHFHS diet. By combining this with prior intra-NAc or intra-VTA infusion of an NPYR-antagonist with behavioral analysis, it will be possible to determine if the Arc->NAc and Arc->VTA NPY projections are directly involved in mediating food-motivated behavior.

Translational notes

The ultimate goal of our research is to contribute to the elucidation of the mechanisms that underlie how diet affects and disrupts the brain in the development of obesity in humans. Knowledge of these disruptions will help to eventually develop targeted therapies that can counteract them. The NPY system is also implicated in the development and maintenance of obesity and the modulation of reward/motivational processes in humans. For example, a body mass index (BMI) above 25 kg/m^2 (overweight and obese subjects) was associated with lower NPY expression in the infundibular nucleus, which is the human homolog to the rat Arc (Alkemade et al., 2012). Also, subjects genetically selected for NPY haplotypes that are associated with low NPY expression in the brain (Zhou et al., 2008), show greater NAc brain activity in response to high-salience stimuli in males as measured with fMRI (Warthen et al., 2019).

The studies in this thesis indicate that diet induces changes in the central NPY system(s) of rats. However, it is not clear whether these changes are adaptive or maladaptive. Unfortunately, the central NPY system cannot be investigated in as much detail in humans as in experimental animals. Indeed, e.g., no positron emission tomography tracers are available to assess this system in the living human brain. Still, studies in humans show that NPY gene variants can predict weight loss, especially in subjects with high-fat intake (X. Lin et al., 2015), and NPY1R and NPY5R gene variations are associated with BMI (P. Li et al., 2014). Though difficult and costly, technically it should be possible to label NPYR-specific ligands to assess their *in vivo* binding profiles in humans, which can be used to assess changes in central NPYRs (Koglin & Beck-Sickinger, 2004). As the development of therapeutic NPYR-specific ligands that

are safe to use in humans is still actively pursued, mechanistic insights into how the central NPY system is affected by diet and obesity in humans can therefore be important.

Thesis conclusions

This thesis describes several fundamental findings on the function and organization of the NPY system during the consumption of a standard low-caloric diet or a high-caloric choice diet. NPY signals via NPY1R in the NAc to stimulate fat intake, and via the NPY1R and NPY5R in the LHA to stimulate carbohydrate/protein intake. Our anatomical studies show that the NAc has a local NPY source and that an afferent Arc NPY projection also contributes to NAc NPY signaling. The origin of NPY in the VTA comes from two afferent projections; Arc NPY neurons and NPY neurons in the C1/A1 areas of the hindbrain. The studies in this thesis also show that the consumption of foods that contain high levels of fat and sucrose can lead to changes in the LHA NPY system of the homeostatic circuitries, and in the dopamine system of the hedonic circuitries.

Future directions

Historically, the Arc->PVN and hindbrain C1/A1->PVN NPY projections have been studied most in relation to homeostatic food intake regulation. In this thesis, we investigated the NPY system(s) in the NAc, LHA and VTA. Our research showed that oftentimes fundamental knowledge on the responsivity or functionality of the NPY system(s) is limited. For instance, NPY1R signaling in the PVN increases food intake, but involvement of the NPY5R has not been accurately established to date (Stanley & Leibowitz, 1984; Yokosuka et al., 1999). Our future directions are therefore aimed at elucidating a number of fundamental properties of the central NPY system(s), such as the NPYRs underlying the effects of NPY on motivation in the VTA, and the role of the different NPYRs in modulating feeding in the NAc. In addition, our initial aims were to also investigate the mechanisms underlying increased sensitivity after long-term fcHFHS diet consumption and the relation between NPY and dopamine signaling. Our findings indicate that the underlying mechanisms should be investigated at the protein level as pharmacological analysis indicated a difference in the functionality of the NPYRs in the LHA, even though gene expression levels were similar (chapter VI). Therefore, pharmacological assays are warranted as well as receptor bindings assays to determine the number of receptors presented on the plasma membrane. In addition, the interconnectedness of the different NPY systems with the local circuitry may provide useful insight in how NPY can eventually modulate feeding-related behaviors.