Brain circuitries in control of feeding behaviors

Focus on Neuropeptide Y

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Chapter III.
Long-term consumption of a free choice high-fat high-sugar diet affects NPY-related gene expression in a brain region-specific manner

Abstract

The central Neuropeptide Y (NPY) system regulates energy balance via NPY neurons in the arcuate nucleus (Arc) of the hypothalamus that sense peripheral signals of energy status, and project to several hypothalamic and extrahypothalamic regions via four NPY receptors (NPYR). NPY also affects the motivation for food when it is infused into areas involved in reward regulation, such as the ventral tegmental area (VTA) and the nucleus accumbens (NAc). Diet-induced obesity dysregulates the NPY system. After one week consumption of a free-choice high-fat high-sugar (fcHFHS) diet, Arc *Npy* mRNA levels are increased. Levels are, however, normalized after four weeks when sensitivity to NPY infusion is increased. The effects of a fcHFHS diet on *Npy* and *Npyr* expression in the reward-related regions is, however, unknown and may play a direct role in the persistent hyperphagia, altered food-motivated behavior, and increased NPY sensitivity observed in rats consuming a fcHFHS diet. Here, our aim was to determine if the expression of the components of the NPY system are altered in the hypothalamus and reward-related regions in diet-induced obesity.

Npy and *Npyr* expression was quantified in the Arc, lateral hypothalamus (LHA), NAc, and VTA after six weeks consumption of a fcHFHS or CHOW control diet by RT-qPCR. mRNA expression was determined 4 hours into the light period and 4 hours into the dark period.

We found that LHA *Npy* expression and NAc *Npy1r* expression were altered after six weeks of fcHFHS diet consumption. LHA *Npy* expression was regulated in an opposite manner to that of CHOW-fed animals; LHA *Npy* was lower in the light period and higher in the dark period in fcHFHS-fed rats. NAc *Npy1r* expression was lower in fcHFHS-fed rats compared to CHOW-fed rats only in the light period.

Introduction

Neuropeptide Y (NPY) regulates energy balance. The central NPY system consists of NPY neurons and their connections in multiple brain regions. NPY neurons in the arcuate nucleus of the hypothalamus (Arc) can sense peripheral signals of energy status (Kohno & Yada, 2012). Accordingly, hypothalamic *Npy* mRNA levels fluctuate with energy status in a circadian manner (Akabayashi, Levin, Paez, Alexander, & Leibowitz, 1994; Jhanwar-Uniyal, Beck, Burlet, & Leibowitz, 1990), and after physiological challenges, such as fasting and refeeding (Hahn et al., 1998; Marks et al., 1992). The Arc NPY neurons project to several hypothalamic and extrahypothalamic regions, including the lateral hypothalamus (LHA), ventral tegmental area (VTA), and nucleus accumbens (NAc) to regulate energy balance via four NPY receptors (NPYR; [Broberger, De Lecea, Sutcliffe, & Hokfelt, 1998; M. C. R. Gumbs et al., 2019; Michel et al., 1998; Sim & Joseph, 1991; van den Heuvel et al., 2015]).

Importantly, the central NPY system is dysregulated in diet-induced obesity (DIO). Hypothalamic NPY expression levels are altered after exposure to obesogenic diets, which has been reported on extensively (for review see [M. C. Gumbs, van den Heuvel, & la Fleur, 2016]). In our model of DIO, the free-choice high-fat high-sugar (fcHFHS) diet, in which rats have *ad libitum* access to chow, fat, a 30% sucrose solution and water (la Fleur et al., 2007), Arc *Npy* expression is already increased after one week of diet consumption (la Fleur et al., 2010), and normalizes to control levels after four weeks of diet consumption (van den Heuvel, Eggels, van Rozen, et al., 2014). However, even when Arc *Npy* levels are normalized, fcHFHS-fed rats continue to show hyperphagia compared to chow-fed controls (van den Heuvel, Eggels, van Rozen, et al., 2014). Strikingly, fcHFHS-fed rats respond more readily to intraventricular NPY infusions than chow-fed controls, indicating that the central NPY system is sensitized (van den Heuvel, Eggels, van Rozen, et al., 2014). The mechanisms underlying the sensitization of the NPY system are, however, currently unknown. One of the hypotheses is that changes in NPYR levels in Arc NPY neuron output structures underlie alterations in NPY sensitivity, such as the PVN and LHA, which are important in the regulation of feeding (Elias et al., 1998; Silverman et al., 1981; Stanley, Daniel, et al., 1985; Stanley et al., 1993). Quantitative autoradiography using a ligand that binds to both the NPY receptor subtype 2 (NPY2R) and 5 (NPY5R), and a ligand that binds specifically to the NPY1R, indicated that NPY2R/NPY5R, but not NPY receptor subtype 1 (NPY1R), was upregulated in the whole hypothalamus after six weeks exposure to a pelleted high-fat high-sugar diet (Widdowson et al., 1997). However, no further specification of region-specific receptor changes in the hypothalamus or of a differentiation between the NPY2R and NPY5R was made.

NPY can also affect food-motivated behavior (Jewett et al., 1995), likely via NPYR in extra-hypothalamic regions (Kishi et al., 2005; R. M. Parker & Herzog, 1999). Importantly, increased motivation to obtain food can contribute to hyperphagia and obesity development. The classic brain circuitry implicated in regulating motivational and reward processing

includes the mesolimbic dopaminergic system, comprised of the VTA, which projects to the NAc (Hernandez & Hoebel, 1988; Meye & Adan, 2014; Wise, 2004). Local infusions of NPY in the VTA and NAc increase the motivation to work for sucrose pellets in normal-weight rats (Pandit et al., 2014a). Accordingly, intra-NAc NPY infusion increases dopamine release in the NAc (Sorensen et al., 2009). After one week of fcHFHS diet consumption, rats show increased motivation to work for sucrose pellets, and increased *Npy* expression in the Arc (la Fleur et al., 2010; la Fleur et al., 2007). The Arc NPY neurons project to both the VTA and NAc (M. C. R. Gumbs et al., 2019; van den Heuvel et al., 2015). The NAc also expresses *Npy* locally (Chronwall et al., 1985; de Quidt & Emson, 1986a), whereas the VTA does not express *Npy* mRNA under normal physiological circumstances, but it may be expressed under non-standard physiological circumstances (M. C. R. Gumbs et al., 2019). Taken together, these findings indicate a role for the NPY system in reward regions in mediating changes in motivation that are also seen in models of DIO. The role of NPY in the mesolimbic system has, however, been understudied. Specifically, it is unknown whether *Npy* and/or *Npyr* expression is altered in the VTA and NAc under conditions when motivation is increased such as DIO.

Our aim was to determine the expression level of *Npy* and the different *Npyr* in hypothalamic and reward-related regions, and if the expression of the components of the NPY system are altered in these regions after six weeks consumption of an obesogenic fcHFHS diet when fcHFHS-fed rats are more sensitive to intracerebroventricular NPY infusion, but Arc *Npy* levels are normalized (van den Heuvel, Eggels, van Rozen, et al., 2014). *Npy* and *Npyr* mRNA expression were determined in the Arc, LHA, VTA, and NAc in male Wistar rats that had access to a fcHFHS or a chow control diet for six weeks. In addition, we assessed this at two time points: 4 hours into the dark period when rats generally consume food, and 4 hours into the light period, when rats are generally fasting.

First, we assessed the regional gene expression of the NPY system-related genes relative to Arc gene expression to provide an indication of the importance of the components of the regional NPY systems. Second, we determined if consumption of a fcHFHS diet lead to changes in NPY system-related gene expression. Finally, as LHA *Npy* expression was altered after six weeks diet consumption, and little is known about local LHA NPY neurons in the rat, we determined the localization of NPY-expressing cell bodies in the LHA by immunocyto-chemistry. We hypothesized a difference between gene expression levels at the different time points, as hypothalamic NPY levels fluctuate in a circadian fashion (Akabayashi et al., 1994; Jhanwar-Uniyal et al., 1990). Also, as we have previously shown that Arc *Npy* levels are normalized after long-term exposure to the fcHFHS diet (van den Heuvel, Eggels, van Rozen, et al., 2014), it was expected that Arc *Npy* levels were not different between both diet groups. In addition, we expected changes in *Npy* or *Npyr* expression that might explain the different responses to NPY infusion in chow- and fcHFHS-fed rats, e.g. changes in the VTA and NAc NPY systems in accordance with increased motivation in fcHFHS-fed rats (la Fleur et al., 2007).

Experimental procedures

Animals and housing

Adult male Wistar rats (Charles River Breeding Laboratories, Sulzfeld, Germany), weighing 270-300 grams at arrival, were housed in temperature- (21 ± 2 ˚C), humidity- (60 ± 5 %) and light-controlled (12:12hr light/dark; lights on 07:00-19:00) rooms with background noise (radio) during the entire experiment. Rats had *ad libitum* access to a container with a standard high-carbohydrate diet (Teklad global diet 2918; 24% protein, 58% carbohydrate, and 18% fat, 3.1 kcal/g, Envigo, Horst, The Netherlands) and a bottle of tap water. The animal ethics committee of the Netherlands Institute for Neuroscience approved all experiments according to Dutch legal ethical guidelines.

Diet intervention

Rats were either placed on an *ad libitum* standard chow diet with tap water (CHOW; see above), or a free-choice high-fat high-sugar (fcHFHS) diet, which allows *ad libitum* intake from a dish of saturated beef tallow (Ossewit/Blanc de Boeuf, Vandemoortele, Belguim; 9 kcal/g), a bottle of 30% w/v sucrose water (mixed from commercial grade sugar and tap water; 1.2 kcal/g), standard chow, and tap water (la Fleur et al., 2007). Food intake was measured at least five times a week, and all components were refreshed twice a week. Experimental infusions began after one week diet of exposure.

Effect of consumption of a fcHFHS diet on NPY system mRNA levels

LHA samples were received from dr. A. Blancas-Velazquez, and have been used in previously published studies (Blancas-Velazquez et al., 2018; M.C.R. Gumbs et al., *accepted*). CHOW- (N = 14) and fcHFHS-fed (N = 14) rats were kept on their respective diets for six weeks, during which food intake and body weight were monitored. Rats were then divided into two groups and euthanized in the light period (11:00), or in the dark period (23:00) by 33%CO_2/66%O_2 anesthesia and immediate decapitation. Brains were rapidly removed, frozen on dry ice and stored at -80 °C. In addition, epididymal fats pads were excised and weighed.

RNA isolation and RT-qPCR procedures have been described before (Blancas-Velazquez et al., 2018; M. C. R. Gumbs et al., 2019). Brains were sectioned coronally on a cryostat at 250 µm and sections were placed in RNAlater (Ambion, Waltham, MA) to be punched according to the Paxinos rat brain atlas (Paxinos & Watson, 2007). The Arc, Bregma -1.72 till -3.00, LHA, Bregma -1.20 till -3.00, VTA, Bregma -4.68 till -6.24, and NAc, Bregma 3.00 till -0.84, were isolated using a 1 mm-diameter blunt punching needle. Punches were placed in 500 µL TriReagent (Qiagen), and homogenized using an Ultra Thurrax homogenizer (IKA, Staufen, Germany). RNA extraction was done by a chloroform extraction followed by RNA purification using the Machery Nagel nucleospin RNA clean-up kit. RNA quality was determined using Agilent RNA nano chips, and was analyzed with a Bioanalyzer (Agilent,

Santa Clara, USA). Only RIN values above 8.50 were included. cDNA synthesis was carried out using equal RNA input for the LHA, VTA, and NAc (300 ng; as measured with Denovix DS11; Denovix, Wilmington) and the transcriptor first-strand cDNA synthesis kit with oligo d(T) primers (04897030001; Roche Molecular Biochemicals, Mannheim, Germany). cDNA synthesis for the Arc was carried out using 125 ng RNA input. cDNA synthesis reactions without reverse transcriptase were used as a control for genomic DNA contamination. RT-qPCR was performed for *Npy*, *Npy1r*, *Npy2r*, *Npy4r*, *Npy5r*, and the reference genes *Ubiquitin-C, Hypoxanthine guanine phosphoribosyl transferase*, *Cyclophilin-A*, and *B-actin* (see Table 1 for all primer sequences), using the SensiFAST no-rox kit (Bioline, London, UK) and Lightcycler® 480 (Roche Molecular Biochemicals). cDNA (2 µL) was incubated in a final reaction volume of 10 µL containing SensiFAST and 25 ng per primer. PCR products were analyzed on a DNA agarose gel for qPCR product size. RT-qPCR quantification was performed using LinReg Software (Ramakers, Ruijter, Deprez, & Moorman, 2003). Samples deviating >5% from the mean PCR efficiency and outliers (Grubb's test) were excluded. Values were normalized using the geometric mean of the three reference genes.

Table 1. Primer sequences.

Gene	NCBI reference number	Forward primer 5'- 3'	Reverse primer 5'- 3'
Npy	NM_012614.2	GACAATCCGGGCGAGGACGC	TCAAGCCTTGTTCTGGGGGCA
Npy1r	NM_001113357.1	TCTCATCGCTGTGGAACGTC	CCGCCAGTACCCAAATGACA
Npy2r	NM_023968.1	TGGTCCTTATACTGGCCTAT	CAGGGTGTTCACCAAAAGAT
Npy4r	NM_031581.2	CATGGACTACTGGATCTTCG	AATGAACCAGATGACCACAA
Npy5r	NM_012869.1	GCCGAAGCATAAGCTGTGGAT	TTTTCTGGAACGGCTAGGTGC
Ubiquitin-C	NM_017314.1	TCGTACCTTTCTCACCACAGTATCTAG	GAAAACTAAGACACCTCCCCATCA
HPRT	NM_012583.2	CCATCACATTGTGGCCCTCT	TATGTCCCCCGTTGACTGGT
Cyclo-A	NM_017101.1	TGTTCTTCGACATCACGGCT	CGTAGATGGACTTGCCACC
ß-actin	NM_031144.3	CATGTACGTAGCCATCCAGGC	CTCTTTAATGTCACGCACGAT

Cyclo-A = Cyclophilin-A, *HPRT* = Hypoxanthine guanine phosphoribosyl transferase, *Npy* = Neuropeptide Y, *Npr1r* = Neuropeptide Y receptor 1, *Npr2r* = Neuropeptide Y receptor 2, *Npy4r* = Neuropeptide Y receptor 4, *Npy5r* = Neuropeptide Y receptor 5

Localization of NPY neurons in the LHA by immunocytochemistry

Procedures for NPY immunocytochemistry have been described before (M. C. R. Gumbs et al., 2019). Briefly, rats were infused with colchicine (N = 6), and perfused with cold saline and 4% paraformaldehyde after i.p. injected pentobarbital. Brains were post-fixed for 24 hours in 4% PFA, cryoprotected in 30% sucrose/phosphate-buffered saline (PBS), and subsequently frozen on dry ice and stored at -80 °C. Cryostat sections (35 µm) were kept at -20 ˚C in cryoprotectant (30% v/v glycerol, 30% v/v glyceraldehyde, 40% v/v 10xPBS).

Free-floating sections were washed in Tris-buffered saline (TBS; 50 mM Tris-Cl, 150 mM NaCl; pH 7.6) and incubated with 1:1,000 rabbit anti-NPY [Niepke 26/11/1988, RRID: AB_2753189, Netherlands Institute for Neuroscience, (Buijs, 1989)] in supermix (0.15 M NaCl, 0.05 M Tris, 0.25% w/v gelatin, 0.5% v/v Triton X-100, pH 7.6 at RT) in a humidified chamber for 1 hour at RT and overnight at 4 ˚C. After TBS washes, sections were incubated 1:500 Alexa Fluor-488 donkey anti-rabbit IgG (H + L) (A21206, Invitrogen) in supermix for 1 hour at RT. After TBS washes, sections were rinsed in PBS and incubated with 1:150 Hoechst (Pure Blue nuclear staining dye 33342; Biorad Laboratories, Hercules) in 1xPBS for 15 min at RT. After TBS washing, sections were coverslipped with Mowiol (10% w/v [Mowiol 4-88; Calbiochem, Merck, Darmstadt, Germany] in 0.1 M Tris-HCL pH 8.5, 25% v/v glycerol), and stored at 4 ˚C in the dark. Every sixth slice from Bregma -1.08 till -4.68 mm was sampled, and the Arc region was used as a positive control. A detailed description of the antibody characterization can be found in (M. C. R. Gumbs et al., 2019).

Fluorescent-stained slices were analyzed using widefield fluorescent microscopy on a Zeiss Axiovert 200M with Plan-NeoFluar objectives at 2.5X (n.a. 0.075) and 5X (n.a. 0.16) magnification to investigate local NPY peptide expression. Fluorescence was excited with an HXP 120 V power supply metalhalide lamp with excitation filters 365/12 nM (Hoechst), 470/40 nM (Alexa Fluor 488), and emission filters >397 nM, and 515/30 nM, respectively. Images were obtained with a black and white camera (ExiAqua, QImaging) and ImageProPlus software (version 6.3, Media Cybernetics, USA), and subsequently analyzed using ImageJ software (version 1.50i, National Institutes of Health, USA).

Statistics and analyses

RT-qPCR quantification was performed using LinReg Software (Ramakers et al., 2003). Samples deviating >5% from the mean PCR efficiency and outliers (Grubb's test) were excluded, which lead to a minimal sample size of N = 5 per group. Values were normalized against *Cyclophilin-A* expression levels, as the other genes were not stably expressed in each region of interest between the diet groups. To compare the expression level of *Npy* and the *Npyr* between regions, data are presented relative to Arc expression levels after correcting for RNA input. To assess the effect of diet and/or time of day on gene expression levels, Two-way ANOVA analyses were performed on *Cyclophilin-A* corrected expression levels, followed by uncorrected Fisher's LSD *post hoc* testing for exploratory analysis. All statistical analyses were performed using Graphpad Prism 8 (version 8.0.2 [263], January 30, 2019). P-values < 0.1 are reported exactly and detailed in supplemental Table 1. All data are mean ± *SEM*.

Results

Regional *Npy* and *Npyr* gene expression

To determine the expression level of *Npy* and *Npyr* in the Arc, LHA, VTA and NAc, we assessed gene expression in CHOW-fed control animals at ZT4, which is summarized in Table 1.

Table 1. Regional *Npy* and *Npyr* gene expression.

Gene	Brain region			
	Arc	**LHA**	**VTA**	**NAc**
Npy	100 %	8.4 ± 0.9 %	2.9 ± 0.2 %	31.2 ± 1.0 %
Npy1r	100 %	7.6 ± 0.7 %	4.2 ± 0.4 %	10.0 ± 0.8 %
Npy2r	100 %	73.3 ± 11.6 %	2.7 ± 0.3 %	9.5 ± 0.6 %
Np4r	100 %	44.3 ± 2.8 %	118.6 ± 12.8 %	n.d.
Npy5r	100 %	12.9 ± 0.7 %	12.5 ± 1.3 %	21.5 ± 2.5 %

All gene expression values are expressed as a percentage of Arc gene expression of the respective gene to enable easier comparison. Arc = arcuate nucleus, LHA = lateral hypothalamic area, NAc = nucleus accumbens, n.d. = not detected, VTA = ventral tegmental area.

Expression levels of *Npy*, *Npy1r*, *Npy2r*, *Npy4r*, and *Npy5r* were highest in the Arc compared to the other brain regions. Compared to the Arc, the NAc and LHA had lower *Npy* expression. *Npy* expression in the VTA was very low compared to the Arc.

The distribution of *Npyr* expression also differed per region. Second to the Arc, *Npy1r* expression was highest in the NAc, and the LHA and VTA contained relatively low *Npy1r* expression. *Npy2r* and *Npy4r* expression showed more variation between the regions. *Npy2r* expression was relatively high in the Arc and LHA, whereas little expression was observed in the NAc, and very little relative *Npy2r* expression was observed in the VTA. Though *Npy4r* expression was low in all of the brain regions examined, *Npy4r* was comparably expressed in the Arc and VTA, with lower levels in the LHA. In addition, *Npy4r* expression was not detectable in the NAc. Lastly, *Npy5r* expression was highest in the NAc after the Arc, whereas the LHA and VTA contained comparable *Npy5r* expression relative to the Arc.

Diet intervention

Prior to diet exposure, animals from both groups showed comparable body weight and caloric intake. After diet exposure, fcHFHS-fed animals increased caloric intake compared to CHOW-fed animals. Epididymal fat pad weight was increased in the fcHFHS group at the end of the experiment compared to that of the CHOW group. Table 2 shows an overview of the characteristics of dietary intervention.

Table 2. Characteristics of dietary intervention.

	Pre-diet BW (gr)¶	End BW (gr)	EWAT/100 gr BW	Caloric intake/day#	Component (%)
CHOW	243 ± 2	410 ± 5	0.5 ± 0.0	72 ± 1.9	na
fcHFHS	243 ± 2	433 ± 7*	0.9 ± 0.1*	98 ± 5.4*	chow 47 ± 3.1 fat 15 ± 1.8 sucrose 39 ± 3.4

¶Body weight presented as mean body weight for the week before diet intervention. #Caloric intake in kcal during experimental diet consumption. BW = body weight, EWAT = epididymal fat pad weight, na = not applicable, * $p < 0.05$ compared to the CHOW group, data are presented as mean ± *SEM*.

Effects of six weeks fcHFHS diet consumption on *Npy*ergic gene expression

Arc Npyergic expression is similar after six weeks of CHOW or fcHFHS diet consumption

Two-way ANOVA analysis revealed similar Arc *Npy* expression in CHOW- and fcHFHS-fed rats at both time points, with no significant main effects of *Diet*, *Time*, or an *Interaction* effect (see Figure 2A, and supplemental Table 1). In addition, Two-way ANOVA analysis revealed that Arc *Npy1r*, *Npy2r*, *Npy4r*, and *Npy5r* expression were not affected by main effects of *Diet*, *Time* or an *Interaction* effect (all $p > 0.05$, see Figures 2B-E, and supplemental Table 1).

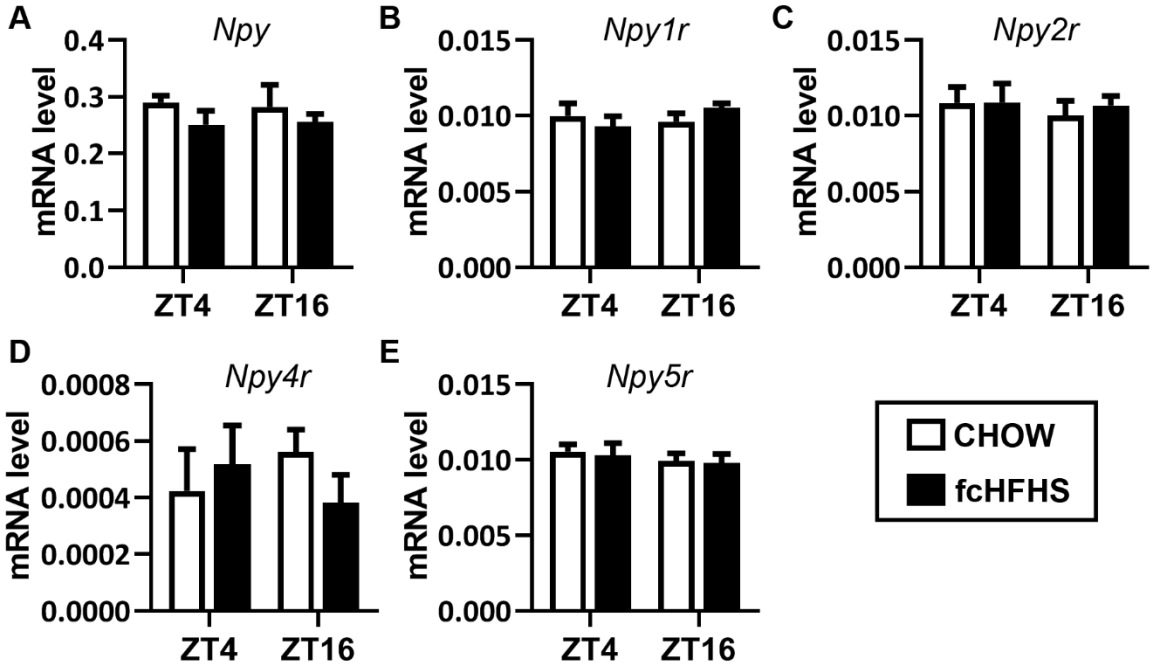

Figure 2. Arc *Npy* and *Npyr* expression levels are unchanged after six weeks of fcHFHS diet consumption or by time of the day. A) Arc *Npy*, **B)** *Npy1r*, **C)** *Npy2r*, **D)** *Npy4r*, and **E)** *Npy5r* mRNA expression were unchanged between CHOW- and fcHFHS-fed rats when measured at ZT4, as well as when measured at ZT16 as determined by Two-way ANOVA analysis**.** See text for details.

LHA Npy expression is altered after six weeks of fcHFHS diet consumption

Two-way ANOVA analysis of LHA *Npy* expression in CHOW- and fcHFHS-fed rats revealed a significant *Interaction* effect ($F_{1,22}$ = 7.36, p = 0.01), and no significant main effects of *Diet* ($F_{1,22}$ = 4.04, p > 0.05), or *Time* ($F_{1,22}$ = 0.00, p > 0.05; see Figure 3A). *Post hoc* analysis revealed that LHA *Npy* mRNA expression was significantly higher in the fcHFHS-fed group *vs.* the CHOW-fed group at ZT16 ($t_{6,6}$ = 3.22, p = 0.004). In addition, *post hoc* analysis showed a trend for lower *Npy* expression at ZT16 *vs.* ZT4 in the CHOW-fed group ($t_{7,6}$ = 1.96, p = 0.06), and a trend for higher *Npy* expression at ZT16 *vs.* ZT4 in the fcHFHS-fed group ($t_{7,6}$ = 3.22, p = 0.07). All other comparisons; p > 0.05.

Two-way ANOVA analysis also revealed that LHA *Npy1r*, *Npy2r*, *Npy4r*, and *Npy5r* expression were not affected by main effects of *Diet*, *Time* or an *Interaction* effect (see Figures 3B-E, and supplemental Table 1).

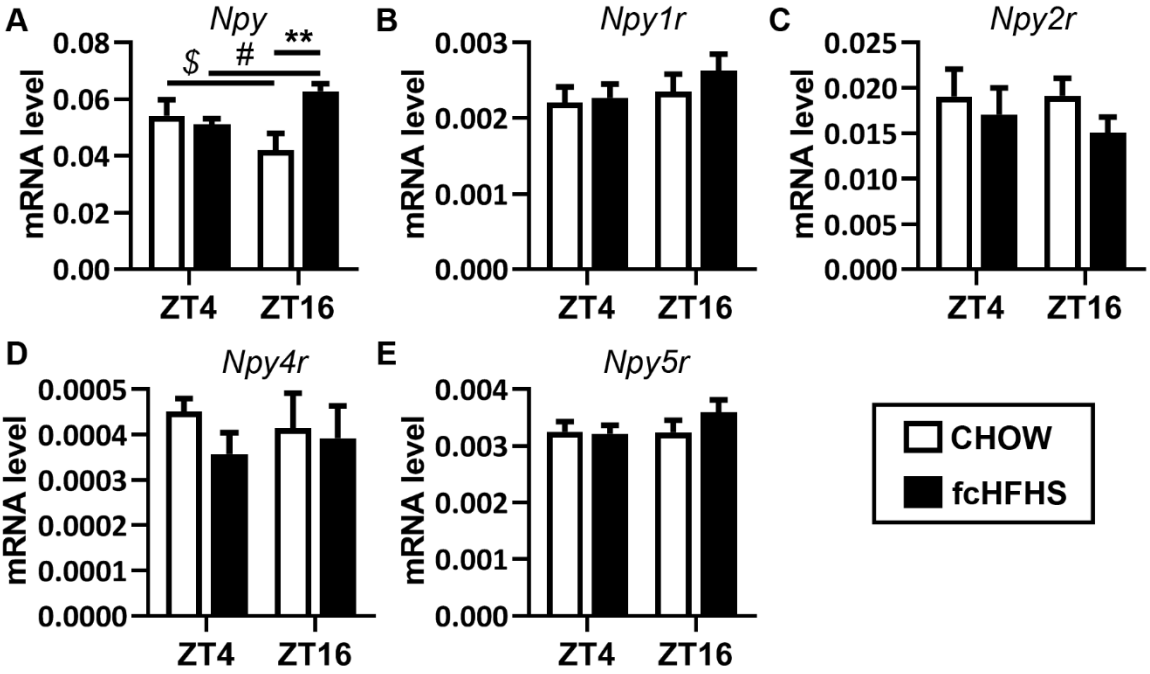

Figure 3. LHA *Npy* expression is increased after six weeks of fcHFHS diet consumption at ZT16. A) Two-way ANOVA analysis revealed a significant *Interaction* effect for LHA *Npy* expression ($F_{1,22}$ = 7.36, p = 0.01). *Post hoc* analysis indicated increased *Npy* expression in fcHFHS- *vs.* CHOW-fed rats at ZT16 ($t_{6,6}$ = 3.22, p = 0.004). In addition, comparison of ZT16 *vs.* ZT4 gene expression showed trends for lower *Npy* expression in the CHOW-fed group ($t_{7,6}$ = 1.96, p = 0.06), and higher *Npy* expression in the fcHFHS-fed group ($t_{7,6}$ = 3.22, p = 0.07). **B)** *Npy1r*, **C)** *Npy2r*, **D)** *Npy4r*, and **E)** *Npy5r* mRNA expression were unchanged in the LHA of CHOW- and fcHFHS-fed rats when measured at ZT4, as well as when measured at ZT16 as determined by Two-way ANOVA analysis**.** ** p < 0.01, $ p = 0.06, # p = 0.07.

VTA Npyergic expression is simliar after six weeks of CHOW or fcHFHS diet consumption

Two-way ANOVA analysis revealed that VTA *Npy* expression was not affected by main effects of *Diet*, *Time* or an *Interaction* in CHOW- and fcHFHS-fed rats (see Figure 4A, and supplemental Table 1).

Two-way ANOVA analysis also revealed that VTA *Npy1r*, *Npy2r*, *Npy4r*, and *Npy5r* gene expression were similar over the different groups, with no main effects of *Diet*, *Time*, or an *Interaction* effect (see Figures 4B-C, and supplemental Table 1).

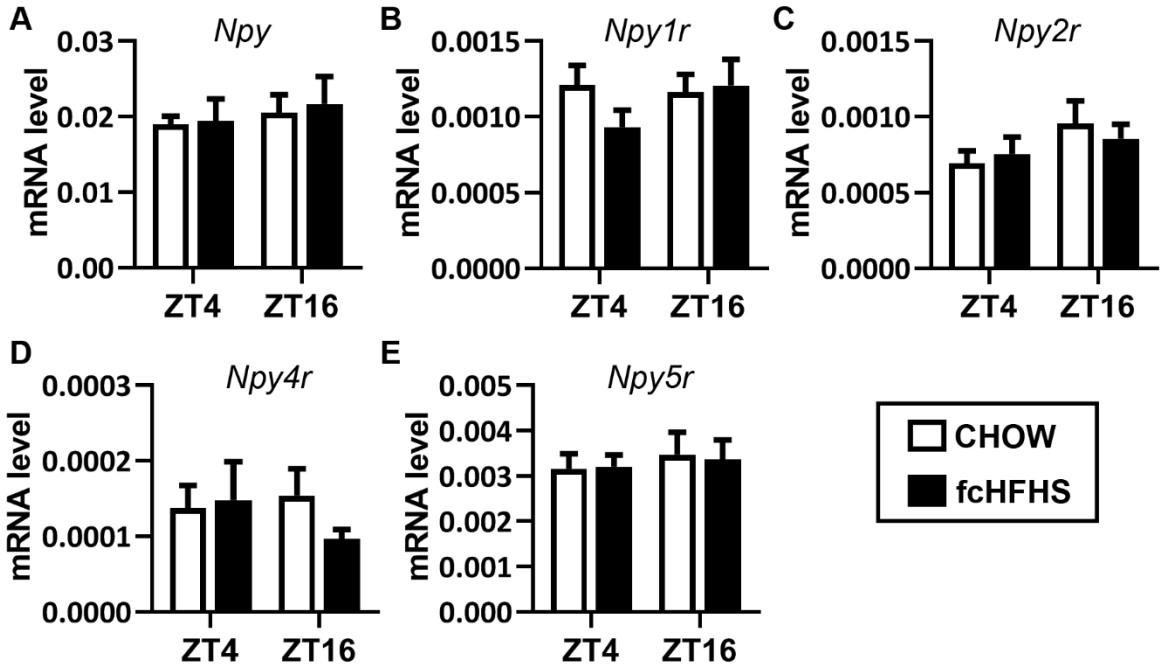

Figure 4. VTA *Npy* and *Npyr* expression levels are unchanged after six weeks of fcHFHS diet consumption or by time of the day. A) VTA *Npy*, **B)** *Npy1r*, **C)** *Npy2r*, **D)** *Npy4r*, and **E)** *Npy5r* mRNA expression is unchanged in fcHFHS- *vs.* CHOW-fed rats when measured at ZT4, as well as when measured at ZT16 as determined by Two-way ANOVA analysis**.** See text for details.

NAc Npy1r expression is altered after six weeks of fcHFHS diet consumption

Two-way ANOVA analysis revealed similar NAc *Npy* expression in CHOW- and fcHFHS-fed rats at both time points, with no significant main effects of *Diet*, *Time* or an *Interaction* effect (see Figure 5A, and supplemental Table 1).

NAc *Npy1r* expression was affected by a significant main effect of *Time* ($F_{1,24}$ = 9.33, p = 0.005), and trends for an effect of *Diet* ($F_{1,24}$ = 4.3, p = 0.05), and an *Interaction* effect ($F_{1,24}$ = 3.09, p = 0.09; see Figure 5B) as revealed by Two-way ANOVA analysis. *Post hoc* analysis revealed a significant decrease in *Npy1r* expression for fcHFHS- *vs.* CHOW-fed rats at ZT4 ($t_{7,7}$ = 2.70, p = 0.01). In addition, *Npy1r* expression was lower at ZT4 *vs.* ZT16 in fcHFHS-fed rats

($t_{7,7}$ = 3.4, p = 0.002). No difference was found in NAc *Npy1r* expression between CHOW- and fcHFHS-fed rats at ZT16 ($t_{7,7}$ = 0.22; p > 0.05), or between NAc *Npy1r* expression at ZT4 and ZT16 in CHOW-fed rats ($t_{7,7}$ = 0.92, p > 0.05).

For NAc *Npy2r*, Two-way ANOVA analysis revealed a trend for an effect of *Time* ($F_{1,24}$ = 3.95, p = 0.06), and no effect of *Diet* ($F_{1,24}$ = 1.38, p > 0.05), or an *Interaction* effect ($F_{1,24}$ = 0.26, p > 0.05). A statistically unjustified *post hoc* analysis indicated a difference in NAc *Npy2r* expression at ZT4 *vs.* ZT16 in fcHFHS-fed rats ($t_{7,7}$ = 2.21, p = 0.04; see Figure 5C). The expression level of *Npy4r* in the NAc did not reach plateau during the qPCR program in samples from both CHOW- and fcHFHS-fed rats (see Figure 5D). Lastly, NAc *Npy5r* expression was not affected by main effects of *Diet*, *Time* or an *Interaction* effect (see Figure 5E and supplemental Table 1).

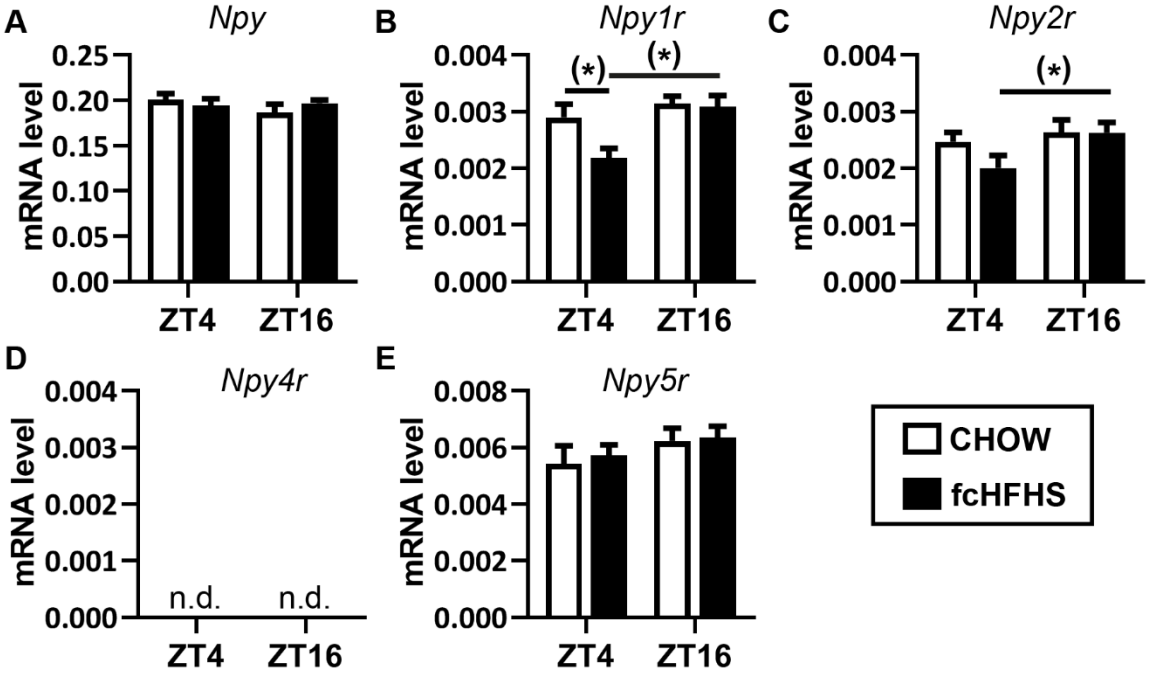

Figure 5. NAc *Npy1r* expression is decreased at ZT4 after six weeks of fcHFHS diet consumption. A) NAc *Npy* expression was similar over all groups. **B)** NAc *Npy1r* mRNA expression was affected by *Time* ($F_{1,24}$ = 9.33, p = 0.005), *Diet (*$F_{1,24}$ = 4.3, p = 0.05), and an *Interaction* effect ($F_{1,24}$ = 3.09, p = 0.09). *Post hoc* analysis revealed a significant difference between *Npy1r* expression in fcHFHS- *vs.* CHOW-fed rats at ZT4 ($t_{7,7}$ = 2.70, p = 0.01), and a difference in expression at ZT4 *vs.* ZT16 for fcHFHS-fed rats ($t_{7,7}$ = 3.4, p = 0.002). **C)** Two-way ANOVA analysis revealed a trend for an effect of *Time* ($F_{1,24}$ = 3.95, p = 0.06) on NAc *Npy2r* mRNA expression. **D)** NAc *Npy4r* was undetectable at both time points and in both diet groups, **E)** NAc *Npy5r* mRNA expression was simliar between fcHFHS- and CHOW-fed rats. n.d. = not detected, (*) p < 0.05 and see text for details.

Immunocytochemical localization of NPY neurons in the LHA

We here show that the LHA expresses *Npy* mRNA, which also varies with diet and time. In studies of feeding behavior, the focus has always been on *Npy* gene expression in Arc. However, our data indicate that the LHA harbors NPY neurons.

According to our RT-qPCR findings, *Npy* is expressed in the LHA to a lower extent compared to the Arc (see Figure 1). Though *Npy*-expressing neurons have been described in the LHA in the mouse and the LHA in the rat (Chronwall et al., 1985; de Quidt & Emson, 1986a; Kosse & Burdakov, 2016; Marston, Hurst, Evans, Burdakov, & Heisler, 2011), the expression patterns differ between these species. We have not been able to replicate the localization of LHA NPY- or *Npy*-expressing neurons mentioned previously, which may also be due to reasons such as advances in the rat brain atlas over the course of 30 years. The most recent and very well executed study of NPY neurons in the rat LHA was limited to the posterior region of the LHA (Abrahamson & Moore, 2001). Therefore, we investigated the expression of NPY throughout the LHA in the male Wistar rat using immunocytochemistry. Throughout the rostro-caudal extent of the LHA, NPY-immunoreactive cell bodies were only located in the LHA in posterior regions of the LHA (see Figures 6A-B, pg. 60). In addition, a relatively high density of NPY-immunoreactive fibers was found surrounding the fornix mainly in the medial part of the LHA in the anteroposterior direction. In addition, we found a band of NPY-immunoreactive cell bodies in a diagonal band in the coronal plane, extending from the tuberal region of the lateral hypothalamus towards the third ventricle and located ventral of the fornix and dorsal of the ventromedial hypothalamus (see Figures 6C-D). Apart from the LHA, NPY-immunoreactive cells were found sporadically in the VMH (data not shown).

Discussion

Here, we show differential expression for the different NPY receptors in the Arc, LHA, VTA and NAc. We also provide details on the distribution of *Npy*-expressing cells throughout the LHA. Our data indicate that six weeks fcHFHS diet consumption specifically affects *Npy* expression in the LHA, and *Npy1r* expression in the NAc in fcHFHS- compared to CHOW-fed rats. Lastly, we show that six weeks consumption of a fcHFHS diet does not affect *Npy* or *Npyr* gene expression in the Arc or VTA compared to consumption of a CHOW diet.

Regional *Npy* mRNA expression

We observed *Npy* mRNA expression in the Arc, NAc and LHA, which is in accordance with data from others, such as the abundance of NPY in the Arc (Chronwall, 1985; Gehlert, Chronwall, Schafer, & O'Donohue, 1987), and the presence of NPY in the NAc (Chronwall et al., 1985; de Quidt & Emson, 1986). We show relatively low levels of *Npy* in the VTA, which is consistent with our previous study that shows that the VTA does not express *Npy* or NPY under normal

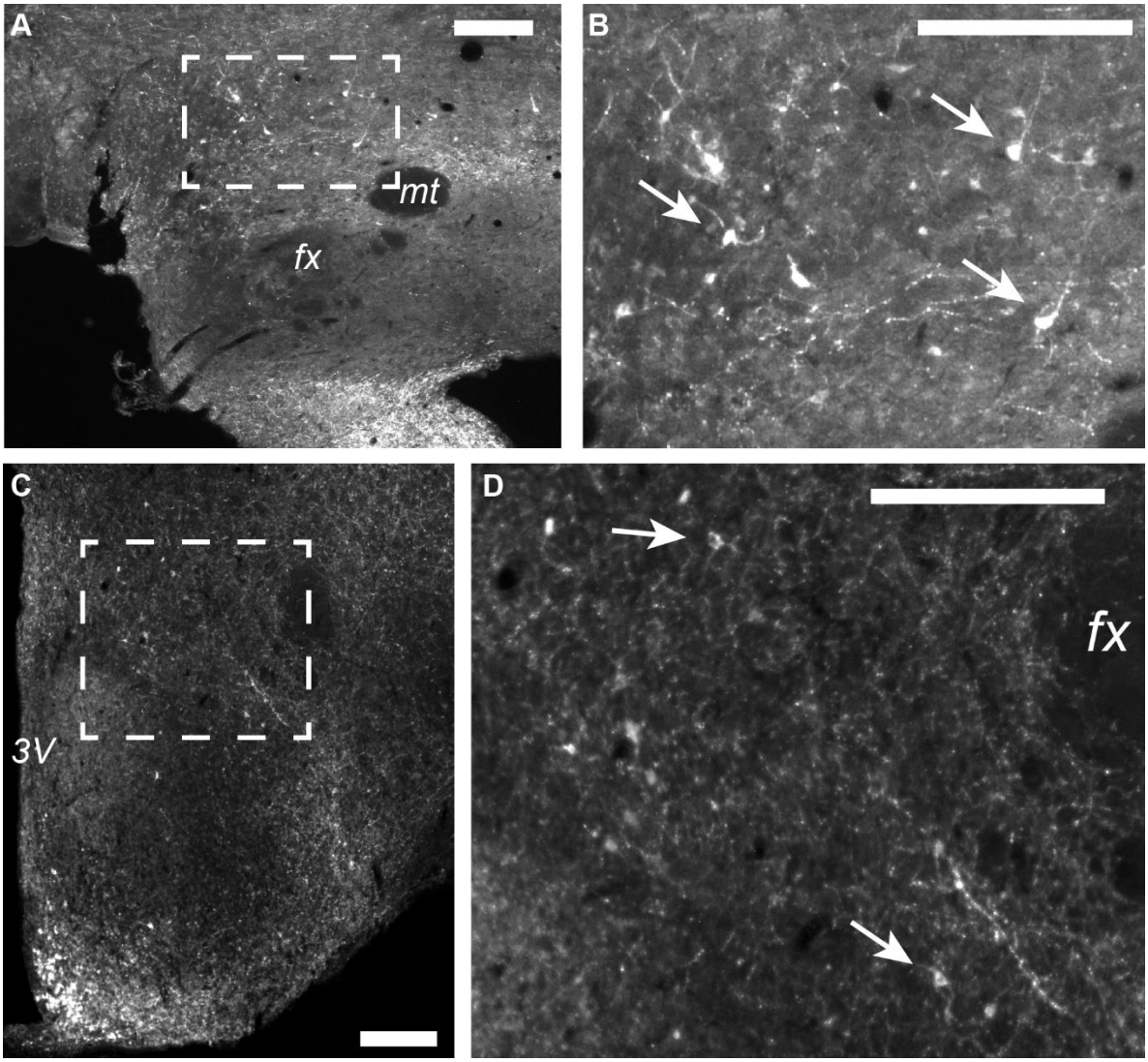

Figure 6. Immunohistochemical localization of NPY-immunoreactive cell bodies in the LHA. A) NPY-immunoreactive cell bodies were located in the posterior part of the LHA at Bregma -4.36 mm, **B)** shows the inset indicated in A, with white arrows pointing to NPY-immunoreactive cell bodies. **C)** At Bregma -2.16 mm, a band of NPY-immunoreactive fibers is seen in an area medial and ventral to the fornix, **D)** shows the inset indicated in C, with white arrows pointing to NPY-immunoreactive cell bodies. All Bregma are according to (Paxinos & Watson, 2007). *3V* = third ventricle, *fx* = fornix, *mt* = mammillothalamic tract, scale bar is 250 µm.

physiological circumstances (M. C. R. Gumbs et al., 2019). Instead, these *Npy* levels likely reflect contamination by *Npy* mRNA in adjacent regions such as the supramammillary nucleus (M. C. R. Gumbs et al., 2019). A relatively low level of *Npy* mRNA expression was found in the LHA. However, as opposed to the VTA, we detected NPY-protein expressing cells in the LHA. The exact localization of LHA NPY/*Npy* neurons had not been reported on extensively in the rat, and differs from the distribution seen in the mouse model expressing green-fluorescent

protein under the NPY promotor (Chronwall et al., 1985; de Quidt & Emson, 1986a; Kosse & Burdakov, 2016). The LHA is mostly considered as an output structure for the Arc NPY system (Elias et al., 1998). Indeed, we found a relatively high density of NPY-immunoreactive fibers surrounding the fornix, and postulate the Arc or hindbrain regions (e.g. the nucleus of the solitary tract, and caudal medullary reticular formation) as likely candidates for input sources (Carstens, Leah, Lechner, & Zimmermann, 1990; Elias et al., 1998). We also localized NPY-immunoreactive cell bodies to the posterior part of the LHA, as previously reported (Abrahamson & Moore, 2001; M. C. R. Gumbs et al., 2019), and additionally localized a band of NPY-immunoreactive cell bodies to an anterior region extending from the tuberal lateral hypothalamus to the third ventricle, which may be partly reckoned to the anterior hypothalamic area according to the rat brain atlas (Paxinos & Watson, 2007).

The effects of six weeks fcHFHS diet consumption *Npy* and *Npyr* gene expression

Arc Npy gene expression is not altered by six weeks of fcHFHS consumption or time of day

Previously, we showed that *Npy* mRNA expression in the Arc was increased after one week of fcHFHS diet consumption, however, expression was similar to CHOW-fed rats after four weeks of fcHFHS diet consumption (la Fleur et al., 2010; van den Heuvel, Eggels, Fliers, et al., 2014). In this study, we also show that after six weeks of fcHFHS diet consumption, no differences are observed in Arc *Npy* expression. In addition, Arc *Npyr* gene expression was also not altered by six weeks of fcHFHS diet consumption. Arc *Npy* and *Npyr* expression also did not differ between the two time points measured. Though the data are not consistent, Arc NPY peptide and *Npy* mRNA have been shown to fluctuate in circadian way in male mice and rat. For example, a peak in *Npy* prior to the start of the dark period has been reported (Akabayashi et al., 1994; Stutz, Staszkiewicz, Ptitsyn, & Argyropoulos, 2007; D. Wang et al., 2017), although an additional peak in the second half of the dark period is also described (Stutz et al., 2007; D. Wang et al., 2017). It has, however, also been described that *Npy* mRNA peaks in the beginning of the light period in tissue punches containing the Arc (Xu, Kalra, Farmerie, & Kalra, 1999), whereas a study in mice report a peak in the early dark period (Kohsaka et al., 2007). We measured *Npy* expression at ZT4 and ZT16, which would be in line with missing the peak at the end of the light period as described by Akabayashi et al. (1994), Stutz et al. (2007), and Wang et al. (2017).

The daily rhythm in LHA Npy expression is altered after six weeks of fcHFHS diet consumption

Interestingly, *Npy* expression in the LHA showed an interaction effect between diet and time, which was explained by increased expression in the fcHFHS- *vs.* the CHOW-fed group in the dark period. In addition, in the CHOW-fed group, LHA *Npy* expression showed a trend to *decrease* from ZT4 to ZT16, whereas in the fcHFHS-fed group, LHA *Npy* expression showed a trend to *increase* from ZT4 to ZT16. These data point to an obesogenic diet-induced alteration

in the rhythm in *Npy* gene expression in the LHA. No studies have looked at circadian rhythmicity in *Npy* mRNA levels specifically in the rat LHA. NPY peptide levels have been measured in the perifornical LHA of male rats, but no rhythm was detected in the LHA (Jhanwar-Uniyal et al., 1990). In female Wistar rats, however, LHA NPY peptide levels were higher three hours after dark onset (i.e. ZT15) compared to three hours before dark onset (i.e. ZT9; [McKibbin, Rogers, & Williams, 1991]). Interestingly, this was specific to the LHA, as other areas within the hypothalamus, such as the Arc, did not show day-night differences. This would point to a day-night effect on the expression of local NPY in the LHA and not on NPY peptide release from afferent fibers originating in the Arc. The female rats showed higher NPY peptide in the dark period, which is opposite to the slightly lower mRNA expression levels we observe in male rats. This could be due to a gender difference, but more studies are needed to confirm this.

Importantly, measuring only two time points does not provide full information on the possible rhythmicity of LHA *Npy* mRNA levels. It may be that other factors, such as food intake, contribute to the differences seen at the beginning of the light period *vs.* the beginning of the dark period. The rhythmicity of LHA *Npy* mRNA expression, as well as other possible factors that may affect LHA *Npy* mRNA expression, thus requires further investigation. In addition, the possible circadian regulation of LHA *Npy* mRNA levels, and the (dys)regulation in DIO warrants further attention to the NPY populations in the LHA of the rat.

VTA and NAc Npy gene expression does not explain changes in motivation in fcHFHS-fed rats

We hypothesized changes in the NPY systems in the reward system based on increased motivation to work for a sugar reward in rats that consumed a fcHFHS diet for two weeks (la Fleur et al., 2007), and the stimulating effects of NPY on motivation (Jewett et al., 1995; Pandit et al., 2014a). However, VTA and NAc *Npy* expression were unaffected by six weeks consumption of a fcHFHS diet, or by time of the day. As mentioned above, we previously concluded that NPY neurons are absent in the VTA under normal physiological circumstances (M. C. R. Gumbs et al., 2019), and extend this here that, also, no changes are observed under obesogenic conditions. We confirm that the NAc expresses *Npy* mRNA and show that *Npy* mRNA expression is not affected by six weeks of fcHFHS diet consumption or by time of the day. Changes in local VTA or NAc *Npy* mRNA thus do not explain changed motivation in animals that consume a fcHFHS diet for six weeks. Therefore, if increased NPY levels play a role in mediating increased motivation in rats consuming a fcHFHS diet, it is more likely to come from an afferent projection (e.g. the Arc or brainstem NPY neurons [Elias et al., 1998; M. C. R. Gumbs et al., 2019]). However, the *Npyr* expression levels are also not changed in the VTA and NAc, which suggests that if fcHFHS-fed rats show increased NPY release in the VTA or NAc, this does not lead to changes in NPYR levels. Alternatively, the NPYR in the VTA and NAc may have undergone posttranslational changes that alter their sensitivity to NPY making it

unlikely to see changes in *Npyr* expression after six weeks diet consumption. Future studies should confirm that the motivation to acquire food is still increased after six weeks fcHFHS diet consumption, and if NPY release or peptide levels are altered in the reward-related regions.

Npyr gene expression is only affected in the NAc after six weeks of fcHFHS consumption

We hypothesized changes in *Npyr* expression after fcHFHS diet consumption given the earlier reported increased sensitivity to NPY when animals are fed an obesogenic diet (Hansen et al., 2004; van den Heuvel, Eggels, van Rozen, et al., 2014). Acute and prolonged consumption of an obesogenic diet increases the excitability of Arc NPY neurons, which might lead to enhanced release in projection areas (Baver et al., 2014; W. Wei et al., 2015). This might subsequently lead to changes in NPYR expression or function. We did not, however, observe changes in *Npyr* mRNA in the Arc, LHA, and VTA after 6 weeks of fcHFHS diet. This is in contrast to earlier reports that showed that NPYR2/NPY5R protein levels increased after 6 weeks consumption of a non-choice high-sugar obesogenic diet in several brain regions, including the Arc and LHA, (Widdowson et al., 1997). It could well be that differences are only observable at the protein level.

We do show that *Npy1r* expression in the NAc was likely affected by diet, depending on the time of the day. We observed a reduction in NAc *Npy1r* expression after consumption of the fcHFHS diet at ZT4, whereas the fcHFHS group did not show decreased NAc *Npy1r* mRNA at ZT16. We have shown that NPY1R in the NAc can mediate fat intake after intra-NAc NPY infusion in fcHFHS-fed rats (van den Heuvel et al., 2015), and that NPY in the NAc is involved in stimulating food-motivated behavior (Pandit et al., 2014a). We here show a clear day-night difference occurring in *Npy1r* mRNA expression which could underlie the clear diurnal rhythm in fat intake, which is more pronounced than the rhythm observed for chow and sugar intake in fcHFHS-fed rats (la Fleur et al., 2014). Whether a rhythm in *Npy1r* in the NAc is casually related to fat intake remains to be studied. We also did not observe a clear upregulation of *Npy1r* in the NAc that might explain the higher sensitivity to NPY in animals consuming a fcHFHS diet for at least four weeks.

Summary

In this study, our aim was to determine if four weeks consumption of a fcHFHS obesogenic diet would affect *Npy* and *Npyr* gene expression in two hypothalamic brain regions, the Arc and LHA, and in two reward-related brain regions, the VTA and NAc. Our data indicate that long-term consumption of a fcHFHS diet leads to alterations in the central NPY systems. Specifically, *Npy* expression in the LHA, and *Npy1r* expression in the NAc are regulated differently compared to the expression in CHOW-fed rats. Moreover, our data indicate that

the role of the NPYR in mediating increased obesogenic diet-induced NPY sensitivity is not apparent in *Npyr* gene expression levels in the Arc, LHA, VTA, or NAc. Further studies could focus on changes in NPY and NPYR peptide levels, and changes in posttranslational processes as NPYR are G-protein-coupled receptors. In addition, future studies should assess the functional implications of these changes for the development and maintenance of obesity, and determine whether they are adaptive or maladaptive. It is known that the central NPY system is disrupted in diet-induced obesity, however the exact disruptions are not elucidated as of yet. Our study specifies distinct diet-induced alterations in regional NPY systems and indicates the existing knowledge gaps on the role of NPY and specific NPYR's in the LHA, VTA, and NAc in mediating food intake and motivational behaviors.

Author contributions

MCR Gumbs designed the RT-qPCR and IHC experiment, AS Blancas-Velazquez performed the animal experiments and provided the samples for RT-qPCR analysis, UA Unmehopa performed laboratory techniques, and MCR Gumbs wrote the manuscript. All authors including JD Mul, and SE la Fleur reviewed the manuscript.

Supplemental Materials

Supplemental Table 1. Statistical details.

Area	Gene	Statistics
Arc	*Npy*	*Diet* $F_{1,23}$ = 1.66, p > 0.05; *Time* $F_{1,23}$ = 0.00, p > 0.05; *Interaction* $F_{1,23}$ = 0.07, p > 0.05
	Npy1r	*Diet* $F_{1,21}$ = 0.03, p > 0.05; *Time* $F_{1,21}$ = 0.45, p > 0.05; *Interaction* $F_{1,21}$ = 1.57, p > 0.05
	Npy2r	*Diet* $F_{1,24}$ = 0.12, p > 0.05; *Time* $F_{1,24}$ = 0.25, p > 0.05; *Interaction* $F_{1,24}$ = 0.09, p > 0.05
	Npy4r	*Diet* $F_{1,24}$ = 0.13, p > 0.05; *Time* $F_{1,24}$ = 0.00, p > 0.05; *Interaction* $F_{1,24}$ = 1.32, p > 0.05
	Npy5r	*Diet* $F_{1,24}$ = 0.09, p > 0.05; *Time* $F_{1,24}$ = 0.83, p > 0.05; *Interaction* $F_{1,24}$ = 0.01, p > 0.05
LHA	*Npy*	*Diet* $F_{1,22}$ = 4.04, p > 0.05; *Time* $F_{1,22}$ = 0.00, p > 0.05; ***Interaction* $F_{1,22}$ = 7.36, p = 0.01** - ZT16: fcHFHS- *vs.* the CHOW-fed group **$t_{6,6}$ = 3.22, p = 0.004** - CHOW-fed group: ZT4 *vs.* ZT16 $t_{7,6}$ = 1.96, p = 0.06 - fcHFHS-fed group: ZT4 *vs.* ZT16 $t_{7,6}$ = 3.22, p = 0.07
	Npy1r	*Diet* $F_{1,22}$ = 0.66, p > 0.05; *Time* $F_{1,22}$ = 1.41, p > 0.05; *Interaction* $F_{1,22}$ = 0.26, p > 0.05
	Npy2r	*Diet* $F_{1,22}$ = 1.48, p > 0.05; *Time* $F_{1,22}$ = 0.16, p > 0.05; *Interaction* $F_{1,22}$ = 0.17, p > 0.05
	Npy4r	*Diet* $F_{1,24}$ = 0.96, p > 0.05; *Time* $F_{1,24}$ = 0.00, p > 0.05; *Interaction* $F_{1,24}$ = 0.37, p > 0.05
	Npy5r	*Diet* $F_{1,24}$ = 0.68, p > 0.05; *Time* $F_{1,24}$ = 0.91, p > 0.05; *Interaction* $F_{1,24}$ = 1.03, p > 0.05
VTA	*Npy*	*Diet* $F_{1,22}$ = 0.08, p > 0.05; *Time* $F_{1,22}$ = 0.46, p > 0.05; *Interaction* $F_{1,22}$ = 0.02, p > 0.05
	Npy1r	*Diet* $F_{1,22}$ = 0.68, p > 0.05; *Time* $F_{1,22}$ = 0.66, p > 0.05; *Interaction* $F_{1,22}$ = 1.31, p > 0.05
	Npy2r	*Diet* $F_{1,22}$ = 0.03, p > 0.05; *Time* $F_{1,22}$ = 2.85, p > 0.05; *Interaction* $F_{1,22}$ = 0.57, p > 0.05
	Npy4r	*Diet* $F_{1,22}$ = 0.43, p > 0.05; *Time* $F_{1,22}$ = 0.24, p > 0.05; *Interaction* $F_{1,22}$ = 0.90, p > 0.05
	Npy5r	*Diet* $F_{1,22}$ = 0.01, p > 0.05; *Time* $F_{1,22}$ = 0.39, p > 0.05; *Interaction* $F_{1,22}$ = 0.03, p > 0.05
NAc	*Npy*	*Diet* $F_{1,24}$ = 0.07, p > 0.05; *Time* $F_{1,24}$ = 0.70, p > 0.05; *Interaction* $F_{1,24}$ = 1.34, p > 0.05
	Npy1r	***Diet* $F_{1,24}$ = 4.3, p = 0.05; *Time* $F_{1,24}$ = 9.33, p = 0.005; *Interaction* $F_{1,24}$ = 3.09, p = 0.09** **- ZT4: fcHFHS- *vs.* CHOW-fed groups $t_{7,7}$ = 2.70, p = 0.01** - ZT16: fcHFHS- *vs.* CHOW-fed groups $t_{7,7}$ = 0.22; p > 0.05 - CHOW-fed group: ZT4 *vs.* ZT16 $t_{7,7}$ = 0.92, p > 0.05 **- fcHFHS-fed group: ZT4 *vs.* ZT16 $t_{7,7}$ = 3.4, p = 0.002**
	Npy2r	*Diet* $F_{1,24}$ = 1.38, p > 0.05; ***Time* $F_{1,24}$ = 3.95, p = 0.06** *Interaction* $F_{1,24}$ = 0.26, p > 0.05 **- fcHFHS-fed group: ZT4 *vs.* ZT16 ($t_{7,7}$ = 2.21, p = 0.04**
	Npy4r	Not applicable
	Npy5r	*Diet* $F_{1,24}$ = 0.19, p > 0.05; *Time* $F_{1,24}$ = 2.29; *Interaction* $F_{1,24}$ = 0.04, p > 0.05

Arc = arcuate nucleus of the hypothalamus, LHA = lateral hypothalamic area, NAc = nucleus accumbens, *Npy* = Neuropeptide Y, *Npy1r*/*Npy2r*/*Npy4r*/*Npy5r* = Neuropeptide Y receptor subtype 1/2/4/5, VTA = ventral tegmental area. All data were tested using Two-way ANOVA analysis and subsequently Fisher's LSD *post hoc* testing.