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Focus on Neuropeptide Y

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Chapter VI.
**Neuropeptide Y signaling in the lateral hypothalamus modulates
diet component selection and is dysregulated
in a model of diet-induced obesity**

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Abstract

The preclinical multicomponent free-choice high-fat high-sucrose (fCHFHS) diet has strong validity to model diet-induced obesity and associated maladaptive molecular changes in the central nervous system. fCHFHS-induced obese rats demonstrate increased sensitivity to intracerebroventricular infusion of the orexigenic Neuropeptide Y (NPY). The brain region-specific effects of NPY signaling on fCHFHS diet component selection are, however, not completely understood. For example, fCHFHS-fed rats have increased intake of chow and fat following intracerebroventricular NPY infusion, whereas NPY administration in the nucleus accumbens, a key hub of the reward circuitry, specifically increases fat intake. Here, we investigated whether NPY infusion in the lateral hypothalamic area (LHA), which is crucially involved in the regulation of food intake, regulates fCHFHS component selection, and if LHA NPY receptor subtypes 1 or 5 (NPYR1/5) are involved.

Male Wistar rats were fed a chow or fCHFHS diet for at least seven days, and received intra-LHA vehicle or NPY infusions in a crossover design. Diet component intake was measured two hours later. Separate experimental designs were used to test the efficacy of NPY1R or NPY5R antagonism to prevent the orexigenic effects of intra-LHA NPY.

Intra-LHA NPY increased caloric intake in chow- and fCHFHS-fed rats. This effect was mediated specifically by chow intake in fCHFHS-fed rats. The orexigenic effects of intra-LHA NPY were prevented by NPY1R and NPY5R antagonism in chow-fed rats, but only by NPY5R antagonism in fCHFHS-fed rats. Thus, NPY signaling has brain region-specific effects on fCHFHS component selection, and LHA NPYR sensitivity is dysregulated during consumption of a fCHFHS diet.

Introduction

The global prevalence of obesity has increased strongly during the last four decades and has reached pandemic levels (Bluher, 2019). Obesity increases the risk for many health impairments, including type 2 diabetes mellitus and cardiovascular diseases, making it a major challenge for individual and public health, and the economy (Bluher, 2019; Stevens et al., 2012; World Health Organization, 2015). The consumption of palatable, energy-dense food, enriched with fats and sugars, dysregulates peripheral and central processes involved in energy homeostasis. Overconsumption of these diets can promote the development of obesity.

Neuropeptide Y (NPY) is a potent regulator of caloric intake and energy homeostasis, (Clark et al., 1985; Loh et al., 2015; Stanley, Chin, et al., 1985). Hypothalamic expression of *Npy* is increased during fasting conditions (Hahn et al., 1998; Marks et al., 1992). NPY neurons in the arcuate nucleus of the hypothalamus integrate central and peripheral information on energy status and relay this information throughout the brain via NPY signaling on four G-protein-coupled NPY receptor subtypes: NPY1R, NPY2R, NPY4R, and NPY5R, to regulate aspects of energy balance (Kohno & Yada, 2012; Michel et al., 1998; Sim & Joseph, 1991). During diet-induced obesity (DIO), the brain NPY circuitry is dysregulated. For example, sensitivity to intraventricular NPY infusion is increased and arcuate nucleus NPY levels are altered, which may occur in a diet component and/or nutrient-specific manner (M. C. Gumbs et al., 2016; Hansen et al., 2004; van den Heuvel, Eggels, van Rozen, et al., 2014; Widdowson et al., 1999).

Administration of NPY in the hypothalamus has classically been associated with increased carbohydrate intake (Stanley, Daniel, et al., 1985; Tempel & Leibowitz, 1990). However, depending on prior dietary preference, it can also increase fat intake (Stanley et al., 1989). Indeed, using the obesogenic free-choice high-fat high-sucrose (fcHFHS) diet, consisting of a container of chow, a dish of beef tallow, a bottle of tap water, and a bottle of 30% sucrose solution, to model DIO in rats (la Fleur et al., 2007; Slomp et al., 2019), we have demonstrated that intracerebroventricular infusion of NPY increases intake of the chow and fat diet components, but not of the sucrose solution (van den Heuvel, Eggels, van Rozen, et al., 2014). Furthermore, the stimulatory effects of NPY on fat intake require NPY1R action in the nucleus accumbens, a key brain region of the reward circuitry (van den Heuvel et al., 2015). These observations indicate that the effects of NPY on fcHFHS diet component selection are mediated in a brain region-specific manner. As NPY administration in the nucleus accumbens did not increase chow intake, it remains to be determined via which brain region NPY signaling can increase chow intake in rats during consumption of a fcHFHS diet.

To date, several studies have used pharmacological approaches to investigate which NPY receptor subtype mediates the orexigenic effects of NPY following intracerebroventri-

cular administration (e.g. [Jain, Horvath, Kalra, & Kalra, 2000; Kanatani et al., 1998; Kanatani et al., 1999; Widdowson et al., 1999; Yokokosuka, Kalra, & Kalra, 1999]). However, no study has investigated these aspects in a brain region-specific manner. The lateral hypothalamic area (LHA) is a key brain region involved in the orexigenic effects of NPY on chow intake (Stanley, Chin, et al., 1985; Stanley et al., 1993; Tiesjema et al., 2007; Tiesjema et al., 2009). No study has, however, investigated which NPY receptor subtype underlies the orexigenic effects of intra-LHA NPY administration. It has thus remained unclear which NPY receptor subtype underlies the effects of intra-LHA NPY on caloric intake and whether this is dysregulated in rats fed a fCHFS diet. Central activation of NPY1Rs or NPY5Rs increases caloric intake (Hu et al., 1996; Kanatani et al., 2000; Mullins et al., 2001), whereas activation of the NPY2R decreases caloric intake (Abbott et al., 2005; Batterham et al., 2002). This makes NPY2Rs unlikely mediators of the orexigenic effects of intra-LHA NPY administration. Central activation of NPY4Rs also increases caloric intake (Campbell et al., 2003; G. Katsuura et al., 2002; Nakajima et al., 1994). However, this receptor subtype has a strong binding preference to pancreatic polypeptide, a ligand from the PP-fold family of ligands, over NPY, making it a less likely mediator of the orexigenic effects of intra-LHA NPY administration (Bard et al., 1995; Gerald et al., 1996; Lundell et al., 1995).

The aim of this study was to determine whether NPY signaling in the LHA regulates fCHFS diet component selection. To do this, we first determined if intra-LHA NPY increases caloric intake in chow-fed and fCHFS-fed rats, and if intra-LHA NPY modulates fCHFS diet component selection. We then assessed the role of the NPY1R and NPY5R in the orexigenic effects of intra-LHA NPY in chow-fed and fCHFS-fed rats, by infusion of the NPY1R antagonist GR231118 or the NPY5R antagonist L-152,804 in the LHA prior to intra-LHA NPY infusion, and measuring caloric intake two hours later. Finally, we also quantified *Npy1r* and *Npy5r* expression in the LHA of chow- and fCHFS-fed rats. This study is the first to determine which NPY receptor subtypes underlie the effect of intra-LHA NPY infusion on caloric intake, and whether this process is dysregulated in rats fed a fCHFS diet. Based on our previous findings in the nucleus accumbens (van den Heuvel et al., 2015), and the LHA-specific findings described in this study, we conclude that NPY can increase intake of chow and/or fat in a brain region-specific manner. We also concluded that LHA NPYR1 sensitivity is lower during consumption of a fCHFS diet.

Experimental procedures

Animals and housing

All experiments were performed in male Wistar rats (Charles River Breeding Laboratories, Sulzfeld, Germany) weighing 270-300 g at arrival at the animal facility of The Netherlands Institute for Neuroscience (Amsterdam, The Netherlands). Rats 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 nutritionally-complete high-carbohydrate diet (chow; 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 committees of the Amsterdam UMC and The Netherlands Institute for Neuroscience approved all experiments according to Dutch legal ethical guidelines.

Stereotactic surgery and fcHFHS diet intervention

One week after arrival, rats were implanted with bilateral cannulas targeting the lateral hypothalamus for the infusion studies. The surgical procedures have been published previously (van den Heuvel et al., 2015). Briefly, rats were anesthetized with an intraperitoneal injection of 80 mg/kg ketamine (Eurovet Animal Health, Bladel, The Netherlands), 8 mg/kg xylazine (Bayer Health Care, Mijdrecht, The Netherlands) and 0.1 mg/kg atropine (Pharmachemie B.V., Haarlem, The Netherlands) and head-fixed in a stereotactic frame. Permanent 26 gauge stainless steel guide cannulas (C315G-SPC 9 mm; PlasticsOne, Bilaney Consultants GmbH, Düsseldorf, Germany) were placed in a 10° angle in the frontal plane with the following coordinates: -2.64 mm anterior/posterior, ± 3.44 mm lateral from Bregma, and -8.2 mm dorsal/ventral below the surface of the skull. Cannulas were secured to the skull using three anchor screws and dental cement, and were occluded by stainless steel dummy's (C315-D; PlasticsOne, Bilaney Consultants GmbH, Düsseldorf, Germany). Immediately after surgery, rats received an analgesic subcutaneously (Carprofen, 0.5 mg/100g body weight) and were housed individually. Rats recovered from surgery until they reached pre-surgical body weight before continuation of the experiments. After recovery, rats received a saline infusion (see *Infusion parameters*) to habituate to the handling procedures, which occurred at least one week before the start of the fcHFHS diet intervention.

Rats had *ad libitum* access to chow and a bottle of tap water, or to a four-component fcHFHS diet. The fcHFHS diet allows simultaneous *ad libitum* access to a dish of saturated beef tallow (Ossewit/Blanc de Boeuf, Vandemoortele, Belgium; 9 kcal/g), a bottle of 30% w/v sucrose solution (mixed from commercial grade sugar and tap water; 1.2 kcal/g), chow pellets, and a bottle of tap water (la Fleur et al., 2007). Intake of diet components was measured at least 5x/week and all components were refreshed 2x/week. Experimental infusions were performed after at least seven days of fcHFHS diet consumption.

Intra-LHA infusions

After seven days of fcHFHS diet consumption, all food components were removed from the cage during the early light phase at 09:00. Intra-LHA infusions were performed at the

beginning of the light phase (between 09:30 and 11:00). Bilateral intra-LHA infusions of 0.3 µg/ 0.3 µL NPY (H6375, Bachem, Germany) in 0.1 mol PBS (PBS; M090001.02NL; Fresenius Kabi GmbH, Zeist, The Netherlands), and 0.3 µg/ 0.2 µL NPY1R-antagonist GR231118 in PBS (sc-361194; Santa-Cruz Biotechnology Inc., Texas, USA; also known as 1229U91 and GW1229), or 1 nmol/ 0.3 µL NPY5R-antagonist L-152,804 (SML0891; Sigma-Aldrich, Missouri, USA) in 8.9% DMSO (D8418; Sigma-Aldrich) or vehicle (0.3 µL 0.1 mol PBS and 8.9% DMSO in 0.1 mol PBS, respectively) were performed using an injector that extended 1 mm below the end of the cannula (C315I, Plastics One, Bilaney Consultants GmbH, Düsseldorf, Germany), and was connected to a 10 µL Hamilton syringe placed in an infusion pump (Harvard Apparatus, Massachusetts, United States of America). Volumes were infused at a rate of 0.3 µL/min and infusion was confirmed by monitoring fluid movement in the tubing via a small air bubble. After infusion, the injector was left in place for 1 min to allow for fluid diffusion. Upon completion of all infusions, all diet components were returned to the animal cage and weighed 2 hours following the intra-LHA infusion of NPY and/or NPYR antagonists.

Exp. 1: Effects of intra-LHA NPY infusion on caloric intake in chow-fed and fchFHS-fed rats

Chow-fed (N = 6) and fchFHS-fed rats (N = 7) were infused with NPY (0.3 µg/ 0.3 µL) or PBS, using a balanced cross-over design with two infusions per week separated by at least two days. At the end of the experiment, rats were perfused, and brains and epididymal fat were isolated for further processing (see section *Perfusion parameters*).

Determination of NPY1R and NPY5R antagonist doses

The doses of the NPY1R and NPY5R antagonists were based on a dose-response experiment performed at the beginning of the dark phase to assess the effect of antagonism of endogenous NPY signaling, which is high at the beginning of the dark phase (Akabayashi et al., 1994). In this exploratory experiment, we assessed the efficacy of NPYR1 antagonism to prevent endogenous NPY-mediated caloric intake by testing intra-LHA infusion of 0, 0.3, 0.45, 1 or 1.5 µg NPY1R antagonist in 0.2 µL 0.1 mol PBS in both diet groups (N = 6/group). At 0.3 µg/0.2 µL, GR231118 did not decrease caloric intake at the start of the dark period, as was seen with 0.45µg/ 0.2 µL and higher doses (see appendix). For the NPY5R antagonist: 0, 0.5, 1, or 3 nmol antagonist in 0.3 µL DMSO were tested in both diet groups (N = 6/group). None of the doses affected intake at the start of the dark period compared to their DMSO control. Therefore, the dosage with the lowest DMSO concentration to not affect intake was chosen; 1 nmol / 0.3 µL 8.9% DMSO (data not shown).

Exp. 2: Effects of intra-LHA NPY1R antagonism on intra-LHA NPY-induced caloric intake

Chow-fed (N = 4) and fchFHS-fed rats (N = 6) were infused intra-LHA with the NPY1R antagonist GR231118 (0.3 µg/0.2 µL) or 0.2 µL PBS 15 min prior to intra-LHA infusion of NPY

(0.3 µg/ 0.3 µL) or 0.3 µL PBS, using a balanced cross-over design with two infusions per week separated by at least two days. Diet component intake was measured 2 hours following the intra-LHA infusions. At the end of the experiment, rats were perfused, and brains and epididymal fat were isolated for further processing (see section *Perfusion parameters*).

Exp. 3: Effects of intra-LHA NPY5R antagonism on intra-LHA NPY-induced caloric intake

Chow-fed (N = 4) and fcHFHS-fed rats (N = 6) were infused with the NPY5R antagonist L-152,804 (0.3 nmol/0.2 µL) or 8.9% DMSO 15 min prior to intra-LHA infusion of NPY (0.3 µg/ 0.3 µL) or PBS, using a balanced cross-over design with two infusions per week separated by at least two days. After completion of all infusions of experiment 3, rats were given access to kaolin (K50001; Research Diets Inc., New Brunswick, USA) in their home cage, next to access to the chow or fcHFHS diet components. Kaolin intake is commonly used as an indication of nausea (Goineau & Castagne, 2016). One day following the introduction of kaolin to the home cage, rats were infused intra-LHA with DMSO/NPY (chow-fed N = 3, fcHFHS-fed N = 3) or NPY5R antagonist/NPY (chow-fed N = 3, fcHFHS-fed N = 4), and caloric intake was measured 2 and 24 hours following intra-LHA infusion. At the end of the experiment, rats were perfused, and brains and epididymal fat were isolated for further processing (see section *Perfusion parameters*).

Table 1. Primer sequences.

Gene	NCBI reference number	Forward primer 5'- 3'	Reverse primer 5'- 3'
<i>Npy1r</i>	NM_001113357.1	TTCATCGCTGTGGAACGTC	CCGCCAGTACCCAAATGACA
<i>Npy2r</i>	NM_023968.1	TGGTCCTTATACTGGCCTAT	CAGGGTGTTCACCAAAGAT
<i>Npy4r</i>	NM_031581.2	CATGGACTACTGGATCTTCG	AATGAACCAGATGACCACAA
<i>Npy5r</i>	NM_012869.1	GCCGAAGCATAAGCTGTGGAT	TTTTCTGGAACGGCTAGGTGC
<i>Ubiq-C</i>	NM_017314.1	TCGTACCTTTCTACCACAGTATCTAG	GAAAATAAGACACCTCCCATCA
<i>HPRT</i>	NM_012583.2	CCATCACATTGTGGCCCTCT	TATGTCCCCCGTTGACTGGT
<i>Cyclo-A</i>	NM_017101.1	TGTTCTTCGACATCACGGCT	CGTAGATGGACTTGCCACC

Cyclo-A = Cyclophilin-A, *HPRT* = Hypoxanthine guanine phosphoribosyl transferase, *Npy1r* = Neuropeptide Y receptor 1, *Npy2r* = Neuropeptide Y receptor 2, *Npy4r* = Neuropeptide Y receptor 4, *Npy5r* = Neuropeptide Y receptor 5, *Ubiq-C* = Ubiquitin-C

Perfusion parameters

At the end of experiments 1, 2, and 3, rats were deeply anesthetized with an intraperitoneal injection of pentobarbital, and the left epididymal fat pad was quickly isolated and weighed. Rats were then transcardially perfused with cold saline followed by 4% PFA in 0.1 mol/L PBS (pH 7.6; 4 °C). Brains were removed and, after 24 hours postfixation in 4% PFA at 4 °C,

cryoprotected in 30% sucrose in PBS at 4°C. Brains were then frozen on dry ice and stored at -80 °C until sectioning. Brains were sectioned coronally on a cryostat at 35 µm. The sections were mounted on Superfrost ++ slides (Merck), stained with thionine (0.5% w/v), and studied with a light microscope to determine whether cannulas were placed in the LHA.

Exp. 4: Effects of consumption of a fCHFS diet on LHA *Npy1r* and *Npy5r* expression

LHA samples were received from dr. A. Blancas-Velazquez, and have been used in a previously published study (Blancas-Velazquez et al., 2018), where chow-fed (N = 6) and fCHFS-fed rats (N = 6) were kept on their respective diets for six weeks, during which caloric intake and body weight was monitored. Rats were euthanized at the beginning of the light period (11:00) using 33%CO₂/66%O₂ anesthesia followed by rapid decapitation. Brains were quickly isolated, frozen on dry ice and stored at -80 °C until usage. Epididymal fat pads were isolated 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. Sections were placed in RNAlater (Ambion, Waltham, MA), and the LHA, Bregma -1.20 till -3.00 mm according to the Paxinos rat brain atlas (Paxinos & Watson, 2007), was 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 (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 reactions without reverse transcriptase were used as a control for genomic DNA contamination. RT-qPCR was performed for *Npy1r*, *Npy2r*, *Npy4r*, *Npy5r*, and the reference genes *Ubiquitin-C*, *Hypoxanthine guanine phosphoribosyl transferase* and *Cyclophilin-A* (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 et al., 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.

Statistical analyses

Only data from rats with correct uni- and bilateral intra-LHA (Bregma -2.28 till -3.72 mm) placements were included in the data analysis. Correct placements were spaced from Bregma -2.28 till -3.72 mm and were contained within an area ventral to the Zona incerta, medial of the internal capsula, and lateral to the dorsomedial and ventromedial hypothalamic nuclei according to the Paxinos rat brain atlas (Paxinos & Watson, 2007). Kilocaloric intake was calculated for each diet item and summed to determine total caloric intake. Body weight, caloric intake over time, and the effect of NPY infusion on intake were analyzed using a mixed-effects model (REML) followed by *post hoc* parametric paired t-tests for component intake comparisons.

Gene expression data complied with normality and equal variance assumptions, which were confirmed with Shapiro-Wilk and Levene's tests for equal variance, respectively. Differences between groups were evaluated using an unpaired Student's t-test. All statistical analyses were performed using Graphpad Prism 8 (version 8.0.2 [263], January 30, 2019). For all cases, a p value < 0.05 was considered significant. All data are presented as mean \pm SEM.

Results

Effects of fcHFHS diet consumption

Before the start of the fcHFHS diet intervention, all rats demonstrated comparable pre-diet body weight and caloric intake. When consuming the fcHFHS diet, rats had significantly greater total caloric intake and larger epididymal fat pads compared to chow-fed controls (see Table 2 for an overview of the effects of the fcHFHS diet).

Table 2. Characteristics of dietary intervention.

	Experiment 1: Intra-LHA NPY	Experiment 2: Y1-antagonist	Experiment 3: Y5-antagonist	Experiment 4: LHA NPYR mRNA
	CHOW / fcHFHS	CHOW / fcHFHS	CHOW / fcHFHS	CHOW / fcHFHS
Pre-diet BW (gr)¶	302 \pm 4 / 302 \pm 5	321 \pm 6 / 325 \pm 5	366 \pm 5 / 366 \pm 7	243 \pm 2 / 243 \pm 2
End BW (gr)	389 \pm 5 / 393 \pm 5	399 \pm 7 / 417 \pm 7	392 \pm 7 / 394 \pm 14	410 \pm 5 / 433 \pm 7*
EWAT/100 gr BW	0.6 \pm 0.0 / 0.9 \pm 0.1*	0.6 \pm 0.0 / 0.8 \pm 0.1*	0.5 \pm 0.0 / 0.8 \pm 0.1*	0.5 \pm 0.0 / 0.9 \pm 0.1*
Kcal intake/day	75 \pm 0.2 / 103 \pm 2.1*	72 \pm 2.1 / 119 \pm 5.5*	79 \pm 1.1 / 115 \pm 2.2*	72 \pm 1.9 / 98 \pm 5.4*

¶Body weight presented as mean body weight for the week before diet intervention. Caloric intake in kcal. BW = body weight, EWAT = epididymal fat pad weight, * p < 0.05 compared to respective CHOW group, data are presented as mean \pm SEM

Intra-LHA NPY infusion increases chow, but not sucrose or fat intake in fCHFHS-fed rats

To assess the role of the LHA in NPY-mediated fCHFHS component selection, NPY was infused intra-LHA in chow- and fCHFHS-fed rats, and caloric intake was measured two hours later. Statistical analysis revealed significant main effects of *Diet* ($F_{1,10} = 33.85$, $p = 0.0002$) and *Infusion* ($F_{1,10} = 19.53$, $p = 0.002$). No significant *Diet* \times *Infusion* interaction effect was observed ($F_{1,10} = 2.845$, $p > 0.05$). Intra-LHA NPY infusion increased intake of chow in both the chow-fed rats ($t_3 = 2.799$, $p = 0.03$) and fCHFHS-fed rats ($t_6 = 3.074$, $p = 0.02$; see Figures 1A-B). Intra-LHA NPY infusion did not significantly affect intake of the sucrose solution ($t_6 = 1.586$, $p > 0.05$) nor of the fat component ($t_6 = 1.159$, $p > 0.05$; see Figures 1C-D).

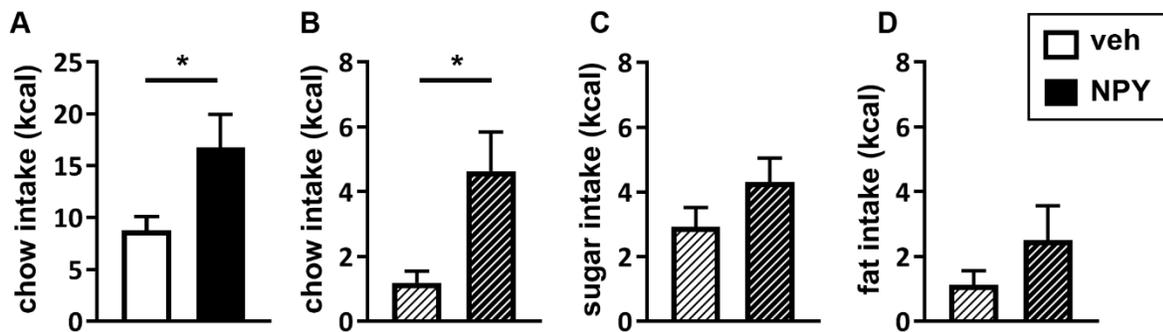


Figure 1. Intra-LHA administration of NPY increases caloric intake in chow- and fCHFHS-fed rats. **A)** In chow-fed control rats, intra-LHA administration of NPY (0.3 μ g/ 0.3 μ L PBS) increases caloric intake of chow during two hours following NPY administration. **B)** In fCHFHS-fed rats, intra-LHA administration of NPY (0.3 μ g/ 0.3 μ L PBS) increases caloric intake of chow, but not of **C)** a 30% sucrose solution or **D)** fat, during two hours following NPY administration. * $p < 0.05$

Intra-LHA NPY1R antagonism prevents intra-LHA NPY-mediated chow intake in chow-fed rats, but not fCHFHS-fed rats

To determine if the effects of intra-LHA NPY on chow intake are mediated by NPY1R, we infused the NPY1R antagonist GR231118 intra-LHA 15 min before intra-LHA NPY infusion in chow- and fCHFHS-fed rats, and measured caloric intake two hours later. Statistical analysis revealed significant main effects of *Diet* ($F_{1,12} = 13.60$, $p = 0.003$), and *Infusion* ($F_{3,36} = 13.66$, $p < 0.0001$), but no *Diet* \times *Infusion* interaction effect ($F_{3,36} = 2.711$, $p > 0.05$). Intra-LHA NPY significantly increased intake of chow in both chow- and fCHFHS-fed rats (veh/veh vs. veh/NPY; $p < 0.05$, see Figures 2A-B). In chow-fed rats, intra-LHA infusion of GR231118 prevented this effect (veh/NPY vs. Y1-anta/NPY; $p = 0.03$). However, for the fCHFHS-fed rats, GR231118 did not prevent the NPY-mediated effects on caloric intake of chow (veh/NPY vs. Y1-anta/NPY; $p > 0.05$, see Figure 2B). We observed no significant effect of NPY or NPY1R antagonism on intake of the sucrose solution ($p > 0.05$), or fat ($p > 0.05$; see Figures 2C-D).

Consistent with the exploratory dose response study (see section *Determination of antagonist doses*), NPY1R antagonism did not significantly affect baseline caloric intake in chow- or fCHFHS-fed rats (veh/veh vs. Y1-anta/veh; $p > 0.05$).

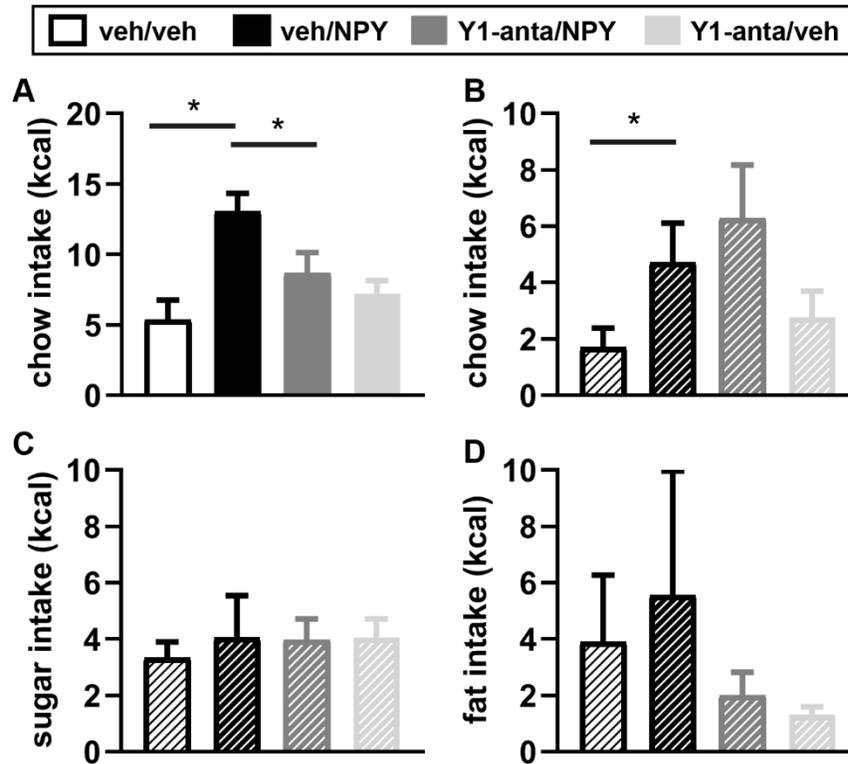


Figure 2. Intra-LHA NPY1R antagonism prevents intra-LHA NPY-mediated chow intake in chow-fed, but not fCHFHS-fed rats. **A)** In chow-fed control rats, intra-LHA administration of NPY (0.3 μg / 0.3 μL PBS) increases caloric intake of chow during two hours following NPY administration, and this is prevented by prior infusion of the NPY1R antagonist GR231118 (Y1-anta; 0.3 μg / 0.3 μL PBS. **B)** In fCHFHS-fed rats, intra-LHA administration of NPY (0.3 μg / 0.3 μL PBS) increases caloric intake of chow, but this is not prevented by prior infusion of the NPY1R antagonist GR231118. **C)** In fCHFHS-fed rats, intra-LHA NPY or NPY1R-antagonist GR231118 infusion does not affect intake of a 30% sucrose solution, or **D)** intake of fat, during two hours following NPY administration.* $p < 0.05$

Intra-LHA NPY5R antagonism prevents intra-LHA NPY-mediated chow intake in both diet groups

To determine if the effects of intra-LHA NPY on chow intake are mediated by NPY5Rs, we infused the NPY5R antagonist L-152,804 in the LHA 15 min prior to intra-LHA NPY infusion in chow- and fCHFHS-fed rats and measured caloric intake two hours later. Statistical analysis revealed significant main effects of *Diet* ($F_{1,8} = 8.523$, $p = 0.02$) and *Infusion* ($F_{3,24} = 7.200$; $p = 0.002$), but not a *Diet x Infusion* interaction effect ($F_{3,24} = 1.176$, $p > 0.05$). In both chow-fed and fCHFHS-fed rats, intra-LHA NPY infusion significantly increased intake of chow (DMSO/PBS

vs. DMSO/NPY, $p = 0.001$), and prior infusion of NPY5R antagonist blocked this effect (DMSO/NPY vs. Y5-anta/NPY; $p = 0.04$; see Figures 3A-B). We observed no significant effect of intra-LHA NPY or NPY5R antagonism on intake of the sucrose solution ($p > 0.05$), or fat ($p > 0.05$; see Figures 3C-D). Consistent with the exploratory dose response study (data not shown), NPY5R antagonism did not significantly affect baseline caloric intake in chow-fed or fCHFHs-fed rats (DMSO/PBS vs. Y5-anta/PBS; $p > 0.05$). In addition, both chow- and fCHFHs-fed rats did not increase kaolin intake after DMSO/NPY vs. Y5-anta/NPY infusion at 2 or 24 hours after infusion (all time points and conditions: intake < 0.1 gr, data not shown), suggesting that intra-LHA infusion of these combinations did not induce nausea.

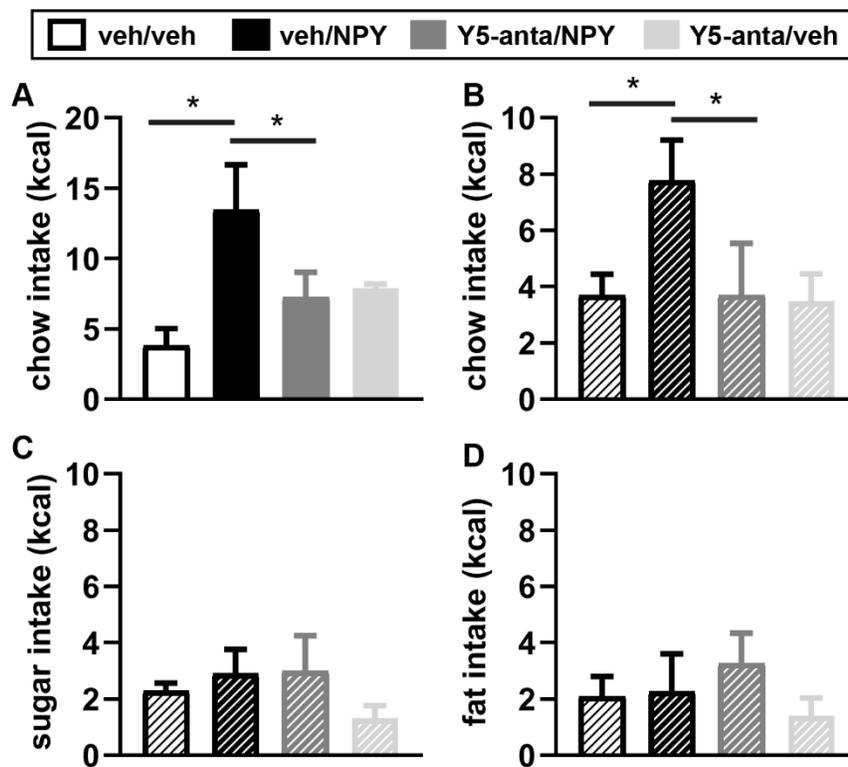


Figure 3. Intra-LHA NPY5R antagonism prevents intra-LHA NPY-mediated chow intake in chow-fed, but not fCHFHs-fed rats. A) In chow-fed control rats, intra-LHA administration of NPY (0.3 μg / 0.3 μL PBS) increases caloric intake of chow during two hours following NPY administration, and this is prevented by prior infusion of the NPY5R antagonist L-152,804 (Y5-anta 1 nmol μg / 0.2 μL 8.89% DMSO). **B)** In fCHFHs-fed rats, intra-LHA administration of NPY (0.3 μg / 0.3 μL PBS) increases caloric intake of chow, and this is also prevented by prior infusion of the NPY5R antagonist L-152,804 (1 nmol μg / 0.2 μL 8.89% DMSO). **C)** In fCHFHs-fed rats, intra-LHA NPY- or NPY5R-antagonist infusion does not affect intake of a 30% sucrose solution, or **D)** intake of fat, during two hours following NPY administration. * $p < 0.05$

Exposure to a fcHFHS diet does not alter LHA *Npy1r* or *Npy5r* expression

To determine whether the difference in the response to intra-LHA NPY1R or NPY5R antagonism in chow- and fcHFHS-fed rats resulted from differences in LHA NPY1R or NPY5R levels, we measured *Npy1r* and *Npy5r* expression in LHA punches from chow- and fcHFHS-fed rats after six weeks of diet consumption. However, no significant differences were observed in LHA *Npy1r* ($t_9 = 0.3697$, $p > 0.05$), or *Npy5r* expression ($t_{11} = 0.8229$, $p > 0.05$; see Figure 4). As this suggested that expression of other LHA NPY receptor subtypes might be modulated by the fcHFHS, we also assessed *Npy2r* and *Npy4r* expression in the LHA punches. However, also no differences in LHA *Npy2r* expression ($t_{11} = 0.2751$, $p > 0.05$), or *Npy4r* expression ($t_{12} = 1.304$, $p > 0.05$) were observed (see Figure 4).

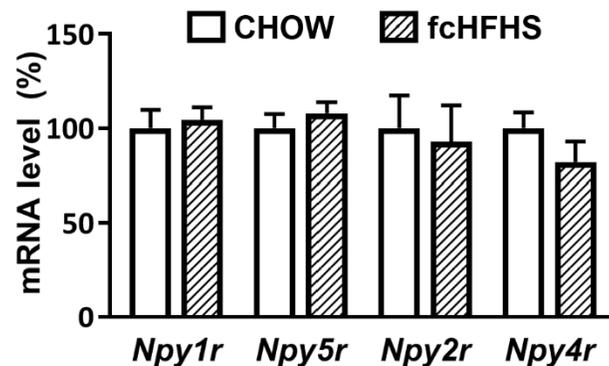


Figure 4. Consumption of a fcHFHS diet for six weeks does not modulate LHA NPY receptor mRNA levels. LHA A) *Npy1r*, B) *Npy5r*, C) *Npy2r*, and D) *Npy4r* mRNA levels were unchanged between chow-fed and fcHFHS-fed rats following six weeks of diet consumption.

Discussion

In this study, we provide evidence that NPY has brain area-specific effects on caloric intake and fcHFHS diet component selection by demonstrating that administration of NPY in the LHA increases chow intake in both chow- and fcHFHS-fed rats. We also determined, for the first time, that NPY receptor subtypes 1 and 5 play an important role in mediating the effects of intra-LHA NPY on caloric intake in both diet groups, and furthermore, that exposure to the obesogenic fcHFHS diet results in lower sensitivity to intra-LHA administration of an NPY1R antagonist, but leaves sensitivity to an NPY5R antagonist unchanged. We also showed that the changes in receptor sensitivity to a receptor-specific antagonist could not be explained by altered gene expression levels. Taken together with the findings previously described by our group (van den Heuvel et al., 2015), we conclude that NPY has brain region-specific effects on dietary selection intake. More specifically, NPY signaling in the nucleus accumbens appears to regulate the specific intake of palatable fat, whereas the lateral hypothalamic area appears to regulate the specific intake of chow.

A role for LHA NPY1R and NPY5R in the regulation of caloric intake

Here, we demonstrate that intra-LHA administration of NPY increases the intake of chow. Our data are in accordance with previously published experiments (Stanley et al., 1993). As intra-LHA administration of NPY elicits the most potent feeding response compared to other brain regions (Stanley et al., 1993; Tiesjema et al., 2007; Tiesjema et al., 2009), the LHA clearly plays a dominant role in the regulation of chow intake. However, we cannot exclude a similar role for other brain regions (Stanley, Chin, et al., 1985). Several experimental paradigms have demonstrated that NPY signaling regulates caloric intake through the NPY1R (MacNeil, 2007). Indeed, administration of NPY1R antagonists in the lateral ventricle consistently reduces caloric intake under physiological circumstances when endogenous NPY levels are high (e.g. fasting; [Kanatani et al., 1996; Widdowson et al., 1999]). Such NPY1R antagonism also prevents the increase in caloric intake induced by intraventricular administration of NPY in chow-fed rats (Jain et al., 2000; Kanatani et al., 1996; Kanatani et al., 1998; Kanatani et al., 1999; Widdowson et al., 1999). Here, we demonstrate that NPY1R antagonism in the LHA prevents caloric intake induced by intra-LHA administration of NPY.

One study has indicated that intraventricular NPY1R antagonism does not reduce spontaneous overnight intake in rats (Widdowson et al., 1999). Finding no effect of intraventricular NPY1R antagonism on overnight intake might be explained by the short-term effects of NPY on caloric intake, which will be occluded by measuring intake after an overnight period. Our exploratory NPY1R antagonist dose response study indicated that intra-LHA NPY1R antagonism lowers caloric intake in the first two hours at the start of the dark period, when rats normally consume many calories and NPY levels are increased (Jhanwar-Uniyal et al., 1990), but only at doses higher than 0.3 µg/ 0.2 µL (Appendix). Together, these findings indicate that LHA NPY1Rs are involved in the physiological regulation of caloric intake.

A role for NPY5Rs in the regulation of caloric intake is less clear. To date, studies investigating the efficacy of NPY5R antagonism to limit caloric intake have produced inconsistent results (MacNeil, 2007). A potential explanation for this might be the difference in specificity of the used NPY5R antagonists. For example, highly effective NPY5R antagonists may show cross-reactivity with other receptors that play a role in the regulation of caloric intake (Della Zuana et al., 2001). Furthermore, in order to assess if NPY5R antagonists were able to prevent spontaneous intake in chow-fed animals, NPY5R antagonists have been administered using various administration routes and doses (Daniels, Grizzle, Wiard, Matthews, & Heyer, 2002; Elliott, Oliver, Hammond, et al., 2003; Gillman et al., 2006; Hammond et al., 2003; Turnbull et al., 2002). Studies that do not observe effects of NPY5R antagonism on caloric intake, often show variable results and often do not report full specificity assays related to the used NPY5R antagonist (Elliott, Oliver, LaFlamme, et al., 2003; Haga et al., 2009; Kakui et al., 2006; G. Li et al., 2008; Mashiko et al., 2008; Moriya et al.,

2009; Sakamoto, Moriya, Haga, et al., 2009; Sakamoto, Moriya, Tsuge, et al., 2009; Sato et al., 2009; H. Takahashi et al., 2009; T. Takahashi et al., 2009; Torrens et al., 2005; Walker et al., 2009; Youngman et al., 2000). Nonetheless, the majority of NPY5R antagonists used, showed no effects on intracerebroventricular NPY-mediated increases in caloric intake. This was even the case when the NPY5R antagonist was also infused intracerebroventricularly or specifically into the paraventricular nucleus of the hypothalamus (Daniels et al., 2002; Gillman et al., 2006; Turnbull et al., 2002). In contrast, L-152,804, the NPY5R antagonist used in this study, has been extensively tested for specificity (see related discussion in *Technical considerations*). In accordance with the current view on NPY5R function, L-152,804 does not affect spontaneous caloric intake or intracerebroventricular NPY-mediated increases in caloric intake (Ishihara et al., 2006; Kanatani et al., 2000). However, it can prevent increases in caloric intake elicited by intracerebroventricular administration of an NPY5R-specific agonist (Ishihara et al., 2006; Kanatani et al., 2000). This suggests a physiological role for NPY5Rs in the regulation of caloric intake during specific physiological conditions. Together with our findings that intra-LHA NPY5R antagonism can block NPY-induced intake, these observations indicate that characterizing the brain region-specific effects of NPY5R antagonism is necessary to provide full insight into the role of the NPY5Rs in feeding behavior.

Consumption of a fcHFHS diet dysregulates NPYR1, but not NPY5R, signaling in the LHA

A limited number of studies have looked into the effects of NPY1R and NPY5R antagonism in animal models consuming palatable high-caloric diets. Here, we show that rats that were fed a fcHFHS diet for a minimal amount of seven days demonstrated a reduction in caloric intake in response to intra-LHA NPY5R antagonism, but did not decrease caloric intake in response to intra-LHA NPY1R antagonism. Oral administration of L-152,804 to mice fed an obesogenic diet led to decreased caloric intake (Ishihara et al., 2006). In contrast, intracerebroventricular administration of an NPY1R antagonist in DIO rats that had been switched back to normal chow, does not reduce caloric intake (Widdowson et al., 1999). Our data appear to be in line with these observations, suggesting that consumption of a high-caloric diet dysregulates central NPYR1, but not NPY5R, function.

Dysregulated function of central NPY1Rs, but not NPY5Rs, could occur via several adaptations. We quantified *Npy1r* and *Npy5r* expression in the LHA and detected no differences between rats fed a standard chow diet or a fcHFHS diet. This suggests that functional changes at the protein level or internalization rates, and not simply changes in receptor expression levels, might explain the differences in behavioral responding to receptor subtype-specific antagonism. Furthermore, *Npy*-expressing neurons in the arcuate nucleus of the hypothalamus are more excitable after consumption of a palatable high-caloric diet (Baver et al., 2014; W. Wei et al., 2015), and *Npy* expression in the arcuate nucleus is higher during consumption of a fcHFHS diet (M. C. Gumbs et al., 2016; la Fleur et al., 2010). Taken

together, these observations suggest greater NPY release in NPY-projection areas, including the LHA, which may result in receptor modification, including glycosylation or phosphorylation states. Notably, NPY1Rs and NPY5Rs have different agonist-driven receptor internalization mechanisms, as internalization of NPY5Rs is relatively insensitive to NPY concentration (Berglund, Schober, Statnick, McDonald, & Gehlert, 2003; S. L. Parker, Parker, Buschauer, & Balasubramaniam, 2003). Moreover, NPY1Rs show a ligand concentration-dependent blockade; a high NPY concentration leads to receptor blockade (S. L. Parker, Parker, Sah, Balasubramaniam, & Sallee, 2007; Sah, Balasubramaniam, Parker, Sallee, & Parker, 2005). Together, these differences may underlie the retention of LHA NPY5R function, but not that of NPY1R function, during consumption of a fCHFHS diet. Lastly, NPY1Rs and NPY5Rs can form heterodimers with each other and with other G protein-coupled receptors (Dinger, Bader, Kobor, Kretzschmar, & Beck-Sickinger, 2003; Gehlert, Schober, Morin, & Berglund, 2007; Kilpatrick, Humphrys, & Holliday, 2015). Therefore, a loss of NPY1R function may represent an increase in the heterodimerization of these receptors at the expense of NPY1R homodimers. Additional studies will have to address whether changes in internalization rates or altered heterodimer composition underlie the differences in behavioral responding to NPY1R- and NPY5R-specific antagonism during consumption of a fCHFHS diet.

Lateral hypothalamic NPY circuitry: relatively unknown

All NPY receptor subtypes are expressed in the LHA (Fetissov, Kopp, & Hokfelt, 2004). The LHA also contains several populations of neurons that are involved in the regulation of caloric intake. However, the role of NPY signaling in these neuronal populations is complex. For example, *Hypocretin*- (also known as orexin) and *pro-melanin concentrating hormone (MCH)*-expressing neurons are two orexigenic neuron populations that are expressed exclusively in the LHA and adjacent areas (Bittencourt et al., 1992; Broberger, De Lecea, et al., 1998; Qu et al., 1996; Sakurai et al., 1998). Both hypocretin and MCH neurons have been functionally linked to the regulation of caloric intake by NPY (Chaffer & Morris, 2002; Ida et al., 2000; Jain et al., 2000; Yamanaka et al., 2000). For example, NPY afferents were found in close apposition to neurons of both populations (Broberger, Johansen, et al., 1998; Elias et al., 1998; Horvath, Diano, & van den Pol, 1999). However, the functional nature of these interactions and their NPY receptor expression profile has not yet been fully characterized.

Other LHA neuronal populations also play a role in feeding behaviors and may be linked with NPY signaling, such as *nitric oxide synthase*-expressing neurons (Fetissov et al., 2003; Morley, Alshaher, Farr, Flood, & Kumar, 1999; Morley, Farr, Sell, Hileman, & Banks, 2011), or GABAergic glutamate-decarboxylase-65-immunoreactive neurons (Jennings et al., 2015; Karnani, Szabo, Erdelyi, & Burdakov, 2013). From our data, it is likely that the NPY1R and NPY5R are mediating NPYs effects via postsynaptic effects, as blocking presynaptic NPY

receptors would be unlikely to suppress feeding elicited by exogenous NPY. Therefore, it is important to know the distribution of NPY receptors on different cell types in the LHA and the functional interaction of NPY with them. In addition, endogenous NPY projections towards the LHA can originate in multiple brain regions including the arcuate nucleus of the hypothalamus and the ventrolateral medulla of the brainstem (Carstens et al., 1990; Elias et al., 1998; Sawchenko et al., 1985). However, it has not yet been investigated which NPYergic source(s) of the LHA mediate the effects on feeding behavior. Further research will have to investigate these open standing questions to determine which effector pathways arise in the LHA to mediate the effect of endogenous NPY release on feeding behavior.

Technical considerations

Here, we used the NPY1R antagonist GR231118 and the NPY5R antagonist L-152,804 to prevent the effects of intra-LHA NPY administration. GR231118 potently antagonizes NPY1R, but also acts as an agonist at NPY4R (E. M. Parker et al., 1998; Schober, Van Abbema, Smiley, Bruns, & Gehlert, 1998). However, it has to be noted that intra-LHA activation of NPY4Rs results in *increased* caloric intake and that NPY4R has a low affinity to NPY (Campbell et al., 2003; Gerald et al., 1996). Thus, the ability of GR231118 to prevent intra-LHA NPY-mediated increases in caloric intake likely results from NPY1R antagonism. The NPY5R antagonist L-152,804 is both very potent and highly selective for the NPY5R, which has been confirmed in NPY5R loss-of-function mice (Ishihara et al., 2006; Kanatani et al., 2000). However, the chemical nature of L-152,804 is associated with low solubility and requires it to be dissolved in DMSO, which can affect caloric intake when administered at a high dose. These disadvantages limited the range of the doses that could be tested in this study. However, we identified and used a dose that was soluble in DMSO, and that was effective in preventing intra-LHA NPY-mediated increases in caloric intake. Notably, intra-LHA administration of 8.9% DMSO did not differ from intake after the saline test infusion prior to exposure to the fcHFHS diet (data not shown). Moreover, administration of both 8.9% DMSO and NPY did not lead to kaolin intake, which is an indication for nausea (see *Results* section; [Goineau & Castagne, 2016]). This is in line with the observations that small volumes of DMSO do not negatively impact caloric intake (Blevins, Stanley, & Reidelberger, 2002). Thus, we conclude that the effect of intra-LHA L-152,804 on NPY-induced intake results from NPY5R antagonism, and is not a result from general nausea.

Summary

Our study is the first to investigate which NPY receptor subtype mediates the effects of intra-LHA administration of NPY on caloric intake by employing a pharmacological approach. Our

results show that intra-LHA NPY increases the intake of chow via the NPY1R and NPY5R, but that these effects are modulated by consumption of a fCHFHS diet. Indeed, in chow-fed control rats antagonism of either LHA NPY1Rs or NPY5Rs prevented the effects of NPY on intake. In fCHFHS-fed rats, however, antagonism of LHA NPY1Rs did not prevent the effects of NPY on intake. This dysregulation of NPY1R function could not be explained by changes in *Npy1r* gene expression. Together with our previous study, where we demonstrate that administration of NPY in the nucleus accumbens results in higher intake of the fat component of the fCHFHS diet specifically (van den Heuvel et al., 2015), our findings show that NPY signaling has brain region-specific effects on dietary selection. Our study provides insight into the neuroanatomical and functional substrates of the NPY brain circuitry under normal physiological circumstances and during consumption of a fCHFHS diet.

Acknowledgments

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MCRG, LE, TK, UAU, and KL performed the experiments. MCRG prepared the manuscript. LE, TK, UAU, JDM and SEIF reviewed the manuscript.