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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 (DIO) 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 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 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 cross-over 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.

Key words: neuropeptide Y, lateral hypothalamus, Neuropeptide Y receptor, GR231118, L-152,804, obesity.

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 (Stevens et al., 2012; World Health Organization, 2015; Bluher, 2019). 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; Stanley et al., 1985a,b; Loh et al., 2015). Hypothalamic expression of *Npy* is increased during fasting conditions (Marks et al., 1992; Hahn et al., 1998). 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

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Abbreviations: DIO, diet-induced obesity; fcHFHS, free choice high-fat high-sucrose; LHA, lateral hypothalamic area; NPY, Neuropeptide Y; NPY1R, Neuropeptide Y receptor 1; NPY5R, Neuropeptide Y receptor 5.

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G-protein coupled NPY receptor subtypes: NPY1R, NPY2R, NPY4R, and NPY5R, to regulate aspects of energy balance (Sim and Joseph, 1991; Michel et al., 1998; Kohno and Yada, 2012). 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 (Widdowson et al., 1999; Hansen et al., 2004; van den Heuvel et al., 2014; Gumbs et al., 2016).

Administration of NPY in the hypothalamus has classically been associated with increased carbohydrate intake (Stanley et al., 1985a,b; Tempel and 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 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 intracerebroventricular administration (e.g. (Jain et al., 2000; Kanatani et al., 1998, 1999; Widdowson et al., 1999; Yokosuka et al., 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 et al., 1985a,b, 1993; Tiesjema et al., 2007, 2009). Similar to the intracerebroventricular studies, no study has 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 fcHFHS 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 (Batterham et al., 2002; Abbott et al., 2005). This makes NPY2Rs unlikely mediators of the orexigenic effects of intra-LHA NPY administration. Central activation of NPY4Rs also increases caloric intake (Nakajima et al., 1994; Katsuura et al., 2002; Campbell et al., 2003). However, this receptor subtype has a strong binding preference to pancreatic polypeptide, a ligand from the NPY family of ligands, over NPY, making it a less likely mediator of the orexigenic effects of intra-LHA NPY administration (Bard et al., 1995; Lundell et al., 1995; Gerald et al., 1996).

The aim of this study was to determine whether NPY signaling in the LHA regulates fcHFHS component selection. To do this, we first determined if intra-LHA NPY increases caloric intake in chow-fed and fcHFHS-fed rats, and if intra-LHA NPY modulates fcHFHS 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 fcHFHS-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-fed and fcHFHS-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 fcHFHS 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 fcHFHS 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 to 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 (12:12 h 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.

Stereotactical 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 stereotaxic 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

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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/100 g 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, Belguim; 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 5×/week and all components were refreshed 2×/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, Massachussets, 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 h following the intra-LHA infusion of NPY and/or NPYR antagonists.

Experiment 1: effects of intra-LHA NPY infusion on caloric intake in chow-fed and fcHFHS-fed rats

CHOW-fed (N = 4) 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 was isolated for further processing (see section *Perfusion parameters*).

Determination of NPY1R and NPY5R antagonist doses

The NPY1R antagonist dose used was based on a dose–response experiment performed just prior to the onset of the dark phase (16:30 p.m). 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 μg, 0.45 μg, 1 μg 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 0.45 μg/0.2 μL and higher doses (Table 1). The NPY5R antagonist dose was chosen based on a dose response performed just prior to the onset of the dark period (16:30 p.m.). To assess the effect of NPYR5 antagonism on endogenous NPY levels, 0, 0.5 nmol, 1 nmol, and 3 nmol NPY5R antagonist in 0.3 μL DMSO were tested in both diet groups (N = 6/group). None of the doses of L-152,804 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 (Table 2). Dose response experiments were carried out at the beginning of the dark phase when the drive to eat is high, and arcuate nucleus NPY levels, and possibly LHA NPY levels, are high. This ensures that the dose does not affect the natural occuring behavioral effects of NPYR activation (Jhanwar-Uniyal et al., 1990; Akabayashi et al., 1994). Experiments were subsequently performed at the beginning of the light phase, when NPY levels are low, to allow a more accurate comparison between both diet groups in reponse to a standard NPY infusion dose.

Table 1. Exploratory dose response for NPY1R antagonist GR231118 in the LHA

NPY1R antagonist (GR231118)	Vehicle	0.3 μg	≥0.45 μg
CHOW	15.4 ± 2.4	18.2 ± 2.9	11.4 ± 1.9
fcHFHS			
– chow	7.9 ± 0.2	8.9 ± 0.0	4.8 ± 0.5
– sucrose water	7.3 ± 0.8	4.9 ± 0.7	5.5 ± 1.3
– fat	6.5 ± 2.1	4.5 ± 2.9	5.0 ± 2.0

Data is included only if cannula placement was within the LHA as defined in the section Statistical Tests (N = 2–4).

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Table 2. Exploratory dose response for NPY5R antagonist L-152,804 in the LHA

NPY5R antagonist (L-152,804)	Vehicle	0.5 nmol	1 nmol	3 nmol
CHOW	20.6 ± 2.1	18.3 ± 2.1	16.0 ± 1.7	14.4 ± 0.7
fcHFHS				
– chow	7.0 ± 0.7	5.8 ± 1.3	6.1 ± 1.4	6.6 ± 1.9
– sucrose water	5.5 ± 1.1	6.3 ± 1.9	6.9 ± 1.5	6.0 ± 1.9
– fat	5.4 ± 1.7	3.1 ± 0.6	1.7 ± 0.9	3.6 ± 0.6

Data is included only if cannula placement was within the LHA as defined in the section Statistical Tests (N = 4–6).

Experiment 2: effects of intra-LHA NPY1R antagonism on intra-LHA NPY-mediated caloric intake

CHOW-fed (N = 6) and fcHFHS-fed rats (N = 7) were infused intra-LHA with the NPY1R antagonist GR231118 (0.3 μg/0.2 μL) or PBS 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. Diet component intake was measured 2 h following the intra-LHA infusions. At the end of the experiment, rats were perfused, and brains and epididymal fat was isolated for further processing (see section *Perfusion parameters*).

Experiment 3: effect of intra-LHA NPY5R antagonist infusion on intra-LHA NPY induced 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 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 and Castagne, 2016). One day following introduction of the kaolin to the homecage, 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 h following intra-LHA infusion. At the end of the experiment, rats were perfused, and brains and epididymal fat was isolated for further processing (see section *Perfusion parameters*).

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 h 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.

Experiment 4: effect of fcHFHS 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 fcHFHS-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%CO_2/66%O_2 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; 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 according to the Paxinos rat brain atlas (Paxinos and 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 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 3 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

Table 3. Primer sequences

Gene	NCBI reference number	Forward primer 5′–3′	Reverse primer 5′–3′
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
Cyclophilin-A	NM_017101.1	TGTTCTTCGACATCACGGCT	CGTAGATGGACTTGCCACC

HPRT = Hypoxanthine guanine phosphoribosyl transferase, *Npy1r* = Neuropeptide Y receptor 1, *Npy2r* = Neuropeptide Y receptor 1, *Npy4r* = Neuropeptide Y receptor 4, *Npy5r* = Neuropeptide Y receptor 5.

deviating >5% from the mean PCR efficiency and outliers (Grubb's test) were excluded. Factor_qPCR [version January 2016.0; (Ruijter et al., 2006)] was used for factor correction, and values were normalized using the geometric mean of the three reference genes.

Statistical tests

Only data from rats with correct uni- and bilateral intra-LHA (Bregma −2.28 till −3.72) placements were included in the data analysis. Correct placements were spaced from Bregma −2.28 till −3.72 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 and Watson, 2007; see Fig. 1). 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 was 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 was 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. Data are presented as mean ± *SEM* in tables. Data are presented as boxplots indicating the median, 1st and 3rd interquartile ranges, and the minimum to maximum values of the data in figures.

RESULTS

Effects of fcHFHS diet consumption

Before 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 4 for an overview of the effects of the fcHFHS diet).

Intra-LHA NPY infusion increases chow, but not sucrose solution 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* × *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 Fig. 2A and 1B). Intra-LHA NPY infusion did not significantly affect intake of the sucrose solution ($t_6 = 1.586$, $p > 0.05$) nor of the fat ($t_6 = 1.159$, $p > 0.05$; see Fig. 2C, D).

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* ($F_{(1,12)} = 13.60$, $p = 0.003$), *Infusion* ($F_{(3,36)} = 13.66$, $p < 0.0001$), but no *Diet* × *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 Fig. 3A and 3B). In chow-fed rats, intra-LHA infusion of GR231118 prevented this effect (veh/NPY *vs.* Y1a/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.* Y1a/NPY; $p > 0.05$, see Fig. 2B). We observed no significant effect of NPY or NPY1R antagonism on intake of the sucrose solution ($p > 0.05$) or the fat ($p > 0.05$; see Fig. 3C, 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.* Y1a/veh; $p > 0.05$).

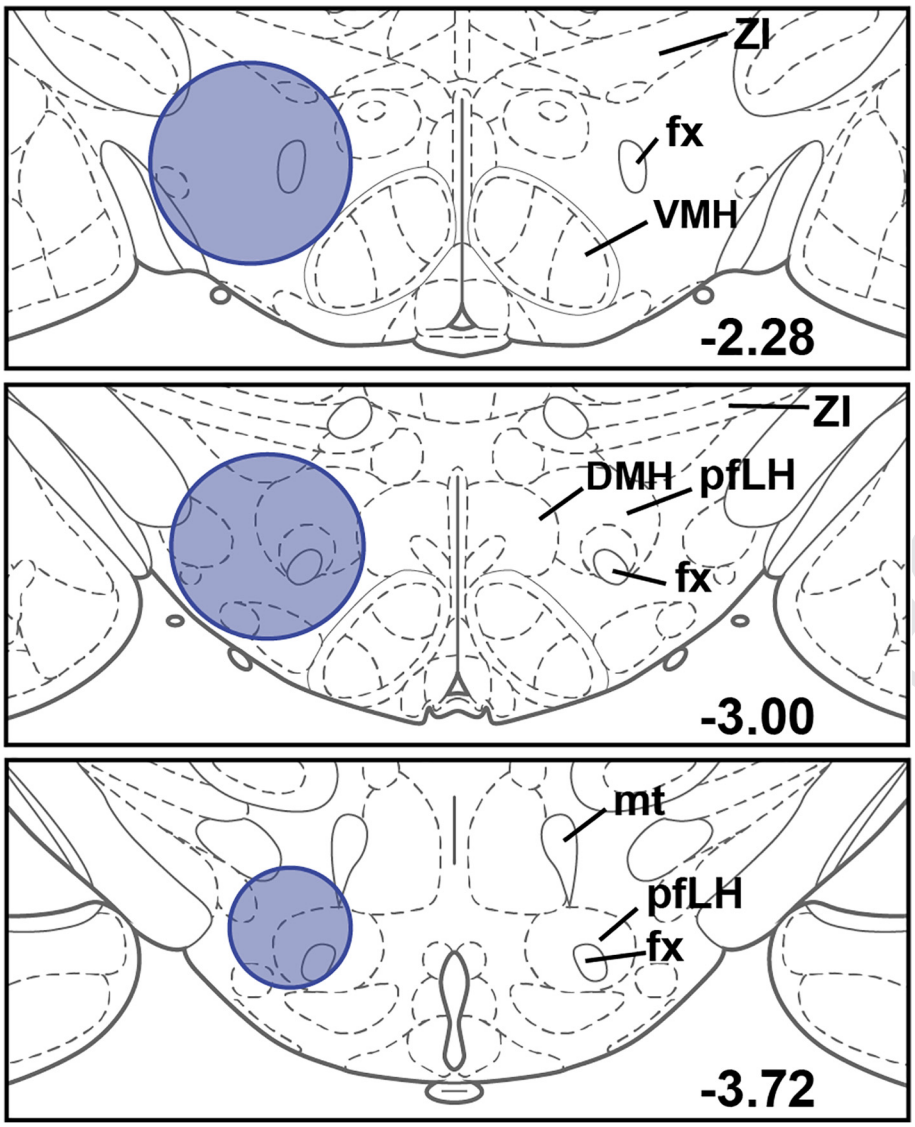

Fig. 1. Cannula placement in the lateral hypothalamic area. Coronal illustrations of the rat lateral hypothalamic area are depicted with the area in which uni- or bilateral cannula tips were identified in blue. Correct placements were spaced from Bregma −2.28 till −3.72 and were contained within an area ventral to the Zona incerta (ZI), medial of the internal capsula, and lateral to the dorsomedial- (DMH) and ventromedial hypothalamic (VMH) nuclei according to the Paxinos rat brain atlas (Paxinos and Watson, 2007). Fx = fornix, mt = mammillothalamic tract, pfLH = perifornical area of the lateral hypothalamus. Numbers indicate the section level relative to Bregma in mm according to Paxinos and Watson (2007).

Intra-LHA NPY5R antagonism prevents intra-LHA NPY-mediated chow intake in chow- and fcHFHS-fed rats

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* × *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.* Y5a/NPY; $p = 0.04$; see Fig. 4A, B). We observed no significant effect of intra-LHA NPY or NPY5R antagonism on intake of the sucrose solution ($p > 0.05$) or the fat ($p > 0.05$; see Fig. 4C, D). Consistent with the exploratory dose response study, NPY5R antagonism did not significantly affect baseline caloric intake in chow-fed or fcHFHS-fed rats (DMSO/PBS *vs.* Y5a/PBS; $p > 0.05$). In addition, both chow- and fcHFHS-fed rats did not increase kaolin intake after DMSO/NPY *vs.* Y5a/NPY infusion at 2 or 24 h 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.

Table 4. Characteristics of dietary intervention

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

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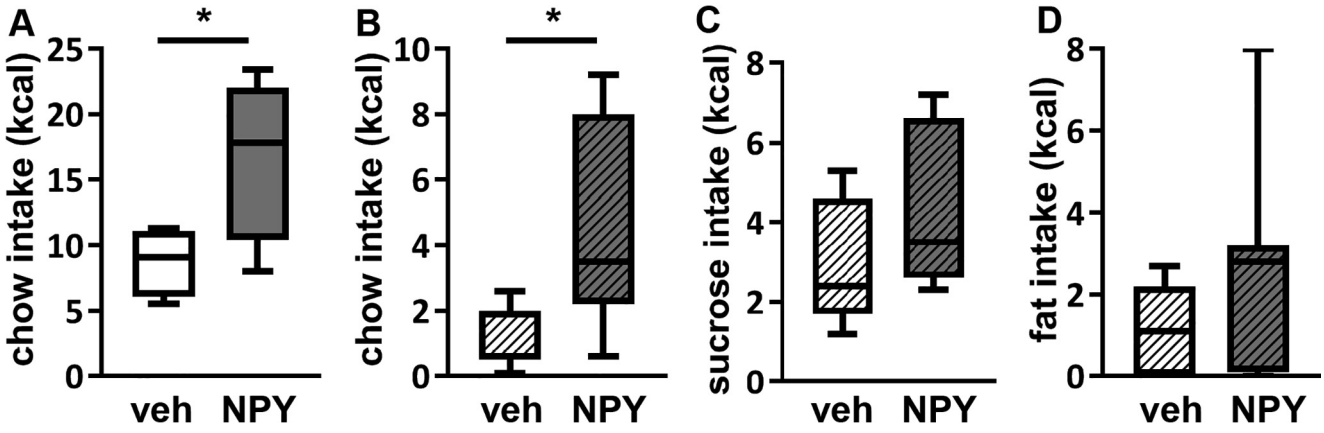

Fig. 2. Intra-LHA administration of NPY increases caloric intake in chow- and fcHFHS-fed rats. (**A**) In chow-fed control rats, intra-LHA administration of NPY (0.3 μg/0.3 μL PBS) increases caloric intake of chow during two hours following NPY administration. (**B**) In fcHFHS-fed rats, intra-LHA administration of NPY (0.3 μg/0.3 μL PBS) increases caloric intake of chow, but not of (**C**) a 30% sucrose solution or (**D**) fat, during two hours following NPY administration. Data are presented as box plots indicating the median, 1st and 3rd interquartile ranges, and the minimum to maximum values of the data. *$p < 0.05$.

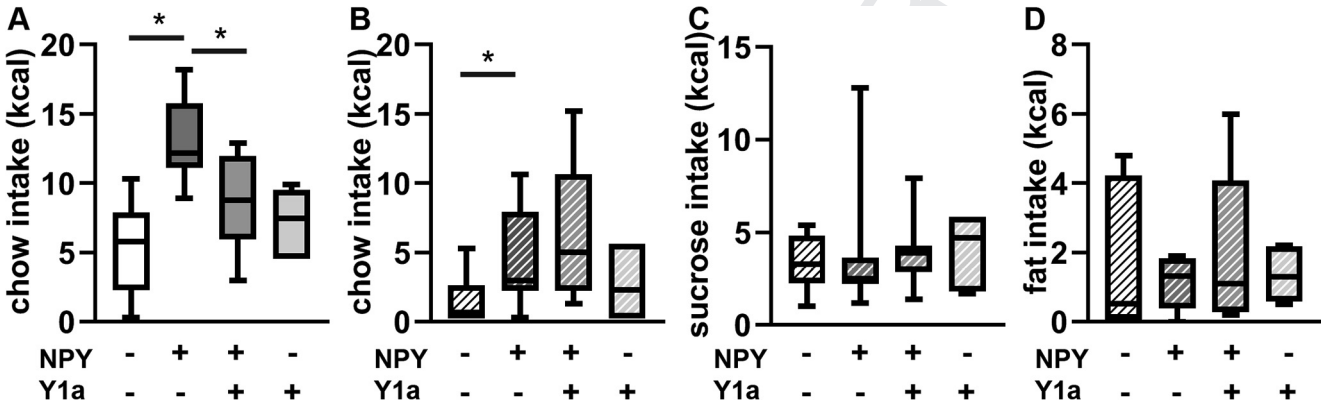

Fig. 3. Intra-LHA NPY1R antagonism prevents intra-LHA NPY-mediated chow intake in chow-fed, but not fcHFHS-fed rats. (**A**) In chow-fed control rats ($N = 6$), Intra-LHA administration of NPY (0.3 μg/0.3 μL PBS) increases caloric intake of chow during two hours following NPY administration, and this is prevented by prior infusion of the NPY1R antagonist GR231118 (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. Data are presented as box plots indicating the median, 1st and 3rd interquartile ranges, and the minimum to maximum values of the data. Y1a = NPY1R antagonist, *$p < 0.05$.

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* ($t_{11} = 0.8229$ $p > 0.05$) expression, see Fig. 5A, B). 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 Fig. 5C, D).

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 these 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

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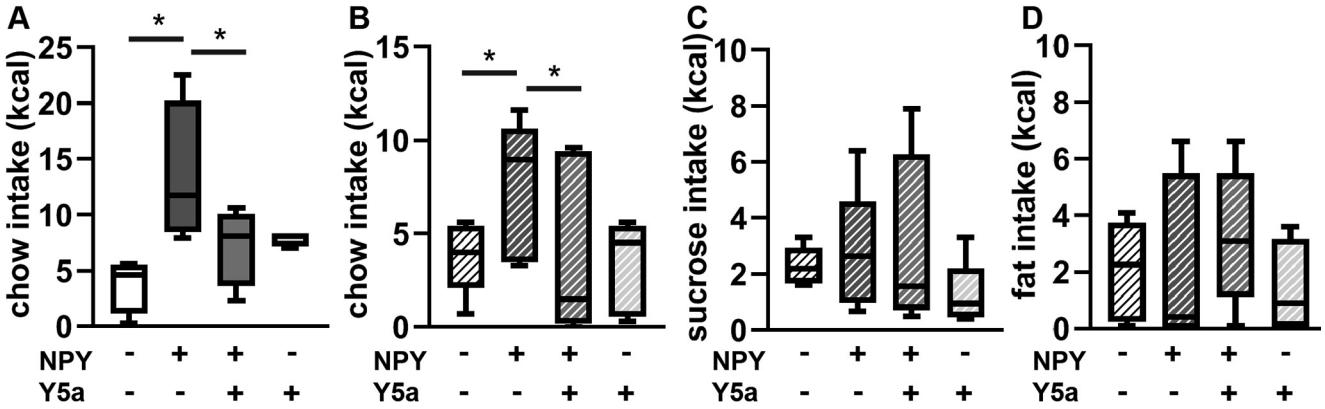

Fig. 4. Intra-LHA NPY5R antagonism prevents intra-LHA NPY-mediated chow intake in chow-fed, but not fcHFHS-fed rats. (**A**) In chow-fed control rats, intra-LHA administration of NPY (0.3 μg/0.3 μL PBS) increases caloric intake of chow during two hours following NPY administration, and this is prevented by prior infusion of the NPY5R antagonist L-152,804 (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 L-152,804 infusion does not affect intake of a 30% sucrose solution or (**D**) intake of fat, during two hours following NPY administration. Data are presented as box plots indicating the median, 1st and 3rd interquartile ranges, and the minimum to maximum values of the data. Y5a = NPY5R antagonist, $*p < 0.05$.

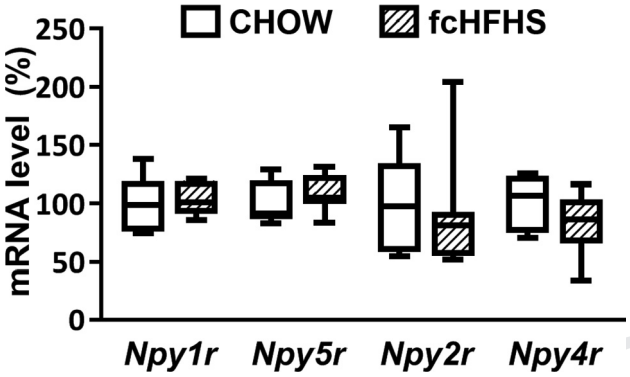

Fig. 5. Consumption of a fcHFHS diet for six weeks does not modulate LHA NPY receptor mRNA levels. LHA (**A**) Npy1r and (**B**) Npy5r (**C**) Npy2r, and (**D**) Npy4r mRNA levels were unchanged between chow-fed and fcHFHS-fed rats following six weeks of diet consumption. Data are presented as box plots indicating the median, 1st and 3rd interquartile ranges, and the minimum to maximum values of the data. See Results section for details.

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 LHA 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, 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 et al., 1985a,b). 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 (Kanatani et al., 1996, 1998, 1999; Widdowson et al., 1999; Jain et al., 2000). 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 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 (data not shown). Together, these findings indicate that LHA NPY1Rs are involved in 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 et al., 2002; Turnbull et al., 2002; Elliott et al., 2003a,b; Hammond et al., 2003; Gillman et al., 2006). 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 (Youngman et al., 2000; Elliott et al., 2003a,b; Torrens et al., 2005; Kakui et al., 2006; Li et al., 2008; Mashiko et al., 2008; Haga et al., 2009; Moriya et al., 2009; Sakamoto et al., 2009a,b; Sato et al., 2009; Takahashi et al., 2009a,b; Walker et al., 2009). 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; Turnbull et al., 2002; Gillman et al., 2006). 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 (Kanatani et al., 2000; Ishihara et al., 2006). However, it can prevent increases in caloric intake elicited by intracerebroventricular administration of an NPY5R-specific agonist (Kanatani et al., 2000; Ishihara et al., 2006). 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 of the consumption of palatable high-caloric diets. Here, we show that rats that were fed a fcHFHS diet for a minimal amount of seven days did demonstrate a reduction in caloric intake in response to intra-LHA NPY5R antagonism, but no decreases in 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. First, we quantified *Npy1r* and *Npy5r* expression in the LHA and detected no differences between rats fed a standard 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. Second, *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; Wei et al., 2015). Furthermore, *Npy* expression in the arcuate nucleus is higher during consumption of a fcHFHS diet (la Fleur et al., 2010; Gumbs et al., 2016). 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 et al., 2003; Parker et al., 2003). Moreover, NPY1Rs show a ligand concentration-dependent blockade; a high NPY concentration leads to receptor blockade (Sah et al., 2005; Parker et al., 2007). 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 et al., 2003; Gehlert et al., 2007; Kilpatrick et al., 2015). Therefore, a loss of NPY1R function may represent an increase in the heterodimerization of these receptors at the expense of NPY1Rs. 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 et al., 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; Qu et al., 1996; Broberger et al., 1998a,b; Sakurai et al., 1998). Both hypocretin and MCH neurons have been functionally linked to the regulation of caloric intake by NPY (Ida et al., 2000; Jain et al., 2000; Yamanaka et al., 2000; Chaffer and Morris, 2002). For example, NPY afferents were found in close apposition to neurons of both populations (Broberger et al., 1998a; Elias et al., 1998,b; Horvath et al., 1999). However, the functional nature of these interactions and their NPY receptor expression profile has not yet been fully characterized. In addition, the interactions between NPY and their LHA neurons targets may vary depending on topographical location, as has been shown for the function of LHA *Hypocretin* neurons (Moorman et al., 2016). Our cannula placement was spaced throughout different

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areas of the LHA, however, future studies should take this into account.

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 (Morley et al., 1999; Fetissov et al., 2003; Morley et al., 2011), or GABAergic glutamate-decarboxy lase-65-immunoreactive neurons (Karnani et al., 2013; Jennings et al., 2015). From our data it is likely that the NPY1R and NPY5R are mediating NPYs effects via post-synaptic 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 ventrolateral medulla of the brainstem (Sawchenko et al., 1985; Carstens et al., 1990; Elias et al., 1998). 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 antagonizes NPY4R (Parker et al., 1998; Schober et al., 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 (Gerald et al., 1996; Campbell et al., 2003). 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 (Kanatani et al., 2000; Ishihara et al., 2006). 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 and Castagne, 2016). This is in line with the observations that small volumes of DMSO do not negatively impact caloric intake (Blevins et al., 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.

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 to the nucleus accumbens results in specific higher intake of the fat component of the fcHFHS diet (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.

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MCRG, LE, TK, UAU, and KL performed the experiments. MCRG prepared the manuscript. LE, TK, UAU, JvdH, KL, JDM and SEIF edited the manuscript. JDM and SEIF supervised the study.

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DATA STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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GLOSSARY

Hyperphagia: feeding beyond homeostatic need
Mediobasal hypothalamus: area of the hypothalamus adjacent to the third ventricle
Orexigenic peptide: peptide that increases feeding

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