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International Journal of Obesity (2008) 32, 1790–1798
 0307-0565/08 $32.00
www.nature.com/ijo

ORIGINAL ARTICLE

SNP analyses of postprandial responses in (an)orexigenic hormones and feelings of hunger reveal long-term physiological adaptations to facilitate homeostasis

M den Hoed, AJPG Smeets, MAB Veldhorst, AG Nieuwenhuizen, FG Bouwman, AG Heidema, ECM Mariman, MS Westerterp-Plantenga and KR Westerterp

Department of Human Biology, Maastricht University, Maastricht, The Netherlands

Background: The postprandial responses in (an)orexigenic hormones and feelings of hunger are characterized by large inter-individual differences. Food intake regulation was shown earlier to be partly under genetic control.
Objective: This study aimed to determine whether the postprandial responses in (an)orexigenic hormones and parameters of food intake regulation are associated with single nucleotide polymorphisms (SNPs) in genes encoding for satiety hormones and their receptors.
Design: Peptide YY (PYY), glucagon-like peptide 1 and ghrelin levels, as well as feelings of hunger and satiety, were determined pre- and postprandially in 62 women and 41 men (age 31 ± 14 years; body mass index 25.0 ± 3.1 kg/m^2). Dietary restraint, disinhibition and perceived hunger were determined using the three-factor eating questionnaire. SNPs were determined in the *GHRL*, *GHSR*, *LEP*, *LEPR*, *PYY*, *NPY*, *NPY2R* and *CART* genes.
Results: The postprandial response in plasma ghrelin levels was associated with SNPs in *PYY* (215G>C, $P<0.01$) and *LEPR* (326A>G and 688A>G, $P<0.01$), and in plasma PYY levels with SNPs in *GHRL* (−501A>C, $P<0.05$) and *GHSR* (477G>A, $P<0.05$). The postprandial response in feelings of hunger was characterized by an SNP–SNP interaction involving SNPs in *LEPR* and *NPY2R* (668A>G and 585T>C, $P<0.05$). Dietary restraint and disinhibition were associated with an SNP in *GHSR* (477G>A, $P<0.05$), and perceived hunger with SNPs in *GHSR* and *NPY* (477G>A and 204T>C, $P<0.05$).
Conclusions: Part of the inter-individual variability in postprandial responses in (an)orexigenic hormones can be explained by genetic variation. These postprandial responses represent either long-term physiological adaptations to facilitate homeostasis or reinforce direct genetic effects.
International Journal of Obesity (2008) **32,** 1790–1798; doi:10.1038/ijo.2008.195; published online 28 October 2008

Keywords: food intake regulation; gastrointestinal hormones; peptides; hypothalamus; nutrition

Introduction

Energy balance is maintained when energy intake matches energy expenditure. To fine-tune energy intake to energy expenditure, an adequate food intake regulation is crucial. Food intake regulation is mainly a behavioral–physiological interaction between the individual and the environment, with hypothalamic receptors responding to peripherally released (an)orexigenic hormones such as leptin, peptide YY (PYY), glucagon-like peptide 1 (GLP-1) and ghrelin. Leptin decreases food intake through the leptin receptor (LEPR) by acting on pro-opiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART) and agouti-related protein (AGRP). POMC expression in the arcuate nucleus is increased by leptin, resulting in the excitation of hypothalamic neurons expressing melanocortin 4 receptor (MC4R) through axons containing α-melanocyte-stimulating hormone (α-MSH).[1] CART is also stimulated by leptin, thereby inhibiting feeding and antagonizing the feeding response induced by the orexigenic neuropeptide Y (NPY).[2] AGRP, on the other hand, is downregulated by leptin,[3,4]

Correspondence: M den Hoed, Department of Human Biology, Maastricht University, Universiteitssingel 50, PO box 616, 6200 MD Maastricht, The Netherlands.
E-mail: m.denhoed@hb.unimaas.nl
Received 28 May 2008; revised 8 September 2008; accepted 9 September 2008; published online 28 October 2008

thereby disinhibiting both the MC4R and the NPY 2 receptor (NPY2R). Furthermore, there is evidence that physiological levels of leptin stimulate GLP-1 release in humans in a dose-dependent manner.[5] GLP-1 binds to G-protein-linked receptors on islet β-cells and decreases food intake in healthy and obese subjects.[6] PYY also reduces appetite and food intake,[7] by stimulating POMC neurons and by inhibiting NPY neurons[8] through the NPY2R.[9] In addition, infusion of PYY decreased plasma ghrelin levels during the fasting period and abolished its preprandial rise.[7] Ghrelin is known to increase food intake through the ghrelin receptor (GHSR) by activating NPY and AGRP-containing neurons in the hypothalamus.[10,11] The interaction between these (an)orexigenic hormones and their receptors is schematically presented in Figure 1.

Fasting levels of several (an)orexigenic hormones were shown to be strongly genotype dependent, suggesting a genetic background in the physiological regulation of food intake. Fasting leptin levels were associated with the −2548G>A single nucleotide polymorphism (SNP) in the promoter region of the leptin gene (*LEP*) and the 668A>G SNP in the leptin receptor gene (*LEPR*);[12–14] fasting plasma PYY levels were associated with a rare variant in the PYY gene (*PYY*)[15] and fasting plasma ghrelin levels appeared to be associated with the −501A>C SNP in the promoter region of the ghrelin gene (*GHRL*).[16]

Cognitive aspects such as dietary restraint, disinhibition and perceived hunger also play a role in the regulation of food intake. A genetic component has been shown in this respect as well, with heritabilities for dietary restraint, disinhibition and perceived hunger ranging from 0.23 to 0.59, 0 to 0.60 and 0.23 to 0.45, respectively.[17–19] The genes involved in these predispositions are yet to be identified.

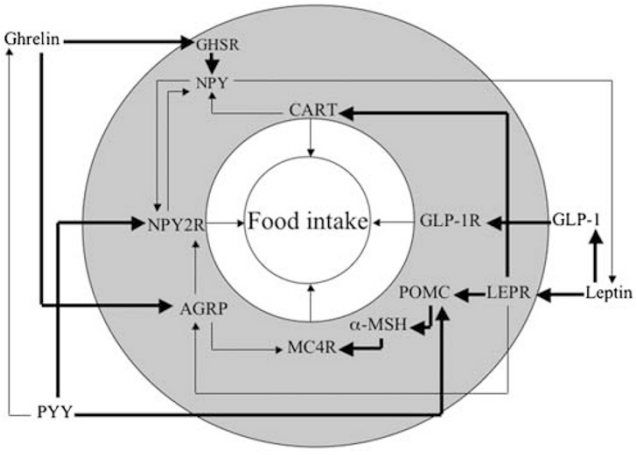

Figure 1 A model for the physiological regulation of food intake by peripherally released (an)orexigenic hormones and their hypothalamic receptors (in gray). α-MSH, alpha-melanocyte-stimulating hormone; AGRP, Agouti-related protein; CART, cocaine and amphetamine-regulated transcript; GHSR, ghrelin receptor; GLP-1, glucagon-like peptide 1; GLP-1R, G-protein-coupled receptor islet β-cell; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; NPY2R, NPY 2 receptor; POMC, pro-opiomelanocortin; PYY, peptide YY; Bold lines represent excitatory effects and thin lines represent inhibitory effects.

Most studies in which the associations between plasma levels of (an)orexigenic hormones and genetic polymorphisms were determined focused on fasting hormone levels. However, humans are in a postprandial state during the largest part of the day, that is, from breakfast onward. Therefore, the postprandial responses of these hormones are potentially important for the physiological regulation of food intake as well. These postprandial responses are characterized by a large inter-individual variability[20] that can only partly be explained by confounders such as body mass index (BMI) and gender.[21,22] We hypothesized that part of this variability can be explained by genetic variation. To increase our understanding of food intake regulation, the objective of this study was to determine whether the postprandial responses in (an)orexigenic hormones and feelings of hunger and satiety are associated with SNPs in genes encoding these proteins and their receptors. Moreover, possible associations between dietary restraint, disinhibition and perceived hunger, measured using the three-factor eating questionnaire, with these SNPs were investigated.

Materials and methods

Data were collected from intervention studies on the effect of proteins and/or protein contents on the postprandial responses in plasma PYY, GLP-1 and ghrelin levels, as well as feelings of hunger and satiety. Subjects came to the university in the morning and received fixed meals in energy balance and according to energy requirement as calculated using the formula of Harris and Benedict.[23] The meals contained 27, 45 and 48% of energy from protein, carbohydrate and fat, respectively.

Subjects

Subjects were recruited using flyers in the university building and advertisements in a local newspaper. Postprandial responses of (an)orexigenic hormones and feelings of hunger and satiety were determined in a total of 103 subjects of Western European descent (62 women and 41 men, age 31 ± 14 years, BMI 25.0 ± 3.1 kg/m^2). For plasma PYY, GLP-1 and ghrelin levels and feelings of hunger and satiety, 60, 78, 70, 102 and 103 subjects, respectively, were available. The study conformed to the standards set by the Declaration of Helsinki, and the Local Ethics Committee approved the study. Subjects provided written informed consent before participating.

Phenotypes

Plasma concentrations of PYY,[3–36] active GLP-1 and active ghrelin were determined pre- and postprandially as described earlier.[24] Feelings of hunger and satiety were determined

using visual analog scales. After adjusting for baseline levels, the postprandial responses of plasma PYY (increases), GLP-1 (increases) and ghrelin (decreases) levels as well as of feelings of hunger (decreases) and satiety (increases) were determined. For all responses, subjects were characterized as responders or non-responders on (1) the initial rate of the response; (2) the absolute response, that is, the difference between the baseline level and the maximal/minimal postprandial level obtained and (3) the postprandial area under the curve, measured until 3–4.5 h postprandially. A high postprandial response can either refer to a strong postprandial increase (for plasma PYY and GLP-1 levels as well as for feelings of satiety), or a strong postprandial decrease (for plasma ghrelin levels and for feelings of hunger). Subjects were dichotomized using gender- and study-specific median values, resulting in an approximately equal number of responders and non-responders per phenotype, meanwhile taking possible gender differences into account.

Attitude towards eating was determined using the Dutch translation of the three-factor eating questionnaire.[25] The first factor measures dietary restraint eating, that is, the control of food intake by thought and will power. The second factor, disinhibition, represents the incidental inability to resist eating cues and can also be seen as inhibition of dietary restraint; in normal weight subjects, it represents emotional eating. Factor three represents the overall feeling of hunger.

DNA isolation and SNP genotyping

Genomic DNA was isolated from peripheral blood leukocytes using the QIAamp blood kit from Qiagen (Amsterdam, The Netherlands). SNPs in genes encoding proteins that exert or mediate (an)orexigenic effects were selected. To ensure an ample number of subjects who are homozygous for the rare alleles, candidate SNPs should have a minor allele frequency in Europeans of at least 25% as indicated by the SNP public database (dbSNP; http://www.ncbi.nlm.nih.gov/SNP). Moreover, only SNPs that were associated earlier with relevant phenotypes, such as BMI or body composition were considered. This resulted in the selection of nine SNPs in eight genes (Table 1): the −501A>C SNP in the promoter region of *GHRL* (rs26802); the silent 477G>A SNP in codon 159 (Arg) in the ghrelin receptor gene (*GHSR*) (rs572169); the −2548G>A SNP in the promoter region of *LEP* (rs7799039); the 326A>G and 668A>G SNPs in *LEPR* resulting in amino-acid substitutions Lys109Arg and Gln223Arg (rs1137100 and rs1137101, respectively); the 215G>C SNP in *PYY* resulting in the non-synonymous amino-acid substitution Arg72Thr (rs1058046); the silent 204T>C SNP in codon 68 (Ser) in *NPY* (rs5574); the silent 585T>C SNP in codon 195 (Ile) in *NPY2R* (rs1047214) and the −156A>G SNP in the promoter region of *CART* (rs35862863).

Genotyping was performed using commercially available TaqMan SNP genotyping assays from Applied Biosystems (Foster City, CA, USA). The procedure was performed according to the manufacturer's protocol and measured on an Applied Biosystems 7900 HT fast real-time PCR system. Allelic calls were determined semi-automatically using the allelic discrimination software of Applied Biosystems.

Table 1 Genotypic and allelic distributions per single nucleotide polymorphism

Gene	*Polymorphism*	*Genotypes*	*F* (n)	*F (%)*	*Allele*	*F (%)*	*HWE*[a]
GHSR	477G>A	GG	38	36.9	G	61.7	0.33
	(Arg159Arg)	GA	51	49.5	A	38.3	
		AA	14	13.6			
GHRL	−501A>C	AA	46	44.7	A	66.0	0.33
		AC	44	42.7	C	34.0	
		CC	13	12.6			
LEPR	326A>G	AA	45	43.7	A	68.9	0.04
	(Lys109Arg)	AG	52	50.5	G	31.1	
		GG	6	5.8			
	668A>G	AA	27	26.2	A	49.0	0.13
	(Gln223Arg)	AG	47	45.6	G	51.0	
		GG	29	28.2			
LEP	−2548G>A	GG	29	28.2	G	51.0	0.23
		GA	47	45.6	A	49.0	
		AA	27	26.2			
PYY	215G>C	GG	38	36.9	G	62.1	0.26
	(Arg72Thr)	GC	52	50.5	C	37.9	
		CC	13	12.6			
NPY	204T>C	TT	30	29.1	T	53.4	0.38
	(Ser68Ser)	TC	50	48.5	C	46.6	
		CC	23	22.3			
NPY2R	585T>C	TT	34	33.0	T	55.3	0.21
	(Ile195Ile)	TC	46	44.7	C	44.7	
		CC	23	22.3			
CART	−156A>G	AA	26	25.2	A	52.9	0.17
		AG	57	55.3	G	47.1	
		GG	20	19.4			

[a]*P*-values obtained from the χ^2 of Hardy–Weinberg equilibrium (HWE). For all single nucleotide polymorphisms, a 100% success rate was accomplished. F, frequency, either absolute (*n*) or relative (%).

Statistical analysis

Data for the postprandial responses were analyzed with dichotomized responses per phenotype ('0' = non-responder and '1' = responder) as dependent variables. For dichotomized responses per phenotype, logistic regression analyses with two dummy variables per SNP were used to determine parameter estimates for the heterozygous and homozygous mutant genotypes. Subjects homozygous for the wild-type allele were used as the reference group. When six or less subjects were identified for the homozygous mutant genotype, carriers of the rare allele were pooled to decrease the chance of false-positive results. This was the case for the

Table 2 Baseline PYY, GLP-1 and ghrelin levels per genotype

Gene	*Polymorphism*	*Genotypes*	*PYY (pmol/l)*	*GLP-1 (pmol/l)*	*Ghrelin (pmol/l)*
GHSR	477G>A	GG	32.8 ± 7.6 (25)	3.3 ± 3.5 (30)	26.0 ± 17.5 (26)
	(Arg159Arg)	GA	31.0 ± 6.6 (30)	2.6 ± 3.6 (39)	27.7 ± 24.6 (32)
		AA	29.4 ± 3.9 (5)	2.4 ± 2.0 (9)	22.8 ± 17.6 (12)
GHRL	−501A>C	AA	32.8 ± 7.3 (25)	2.0 ± 2.8 (32)	26.4 ± 21.8 (31)
		AC	32.2 ± 5.9 (25)	3.5 ± 3.8 (34)	23.4 ± 14.7 (31)
		CC	27.4 ± 7.1 (10)	3.1 ± 3.6 (12)	36.4 ± 34.2 (8)
LEPR	326A>G	AA	32.0 ± 6.9 (31)	3.2 ± 3.9 (38)	26.5 ± 19.3 (27)
	(Lys109Arg)	AG	30.6 ± 6.7 (25)	2.7 ± 3.0 (36)	26.9 ± 22.2 (41)
		GG	35.2 ± 8.3 (4)	1.1 ± 0.3 (4)	08.8 ± 2.9 (2)
	668A>G	AA	32.0 ± 7.3 (21)	3.4 ± 4.0 (26)	30.4 ± 21.4 (19)
	(Gln223Arg)	AG	30.6 ± 6.6 (29)	2.2 ± 2.5 (35)	25.5 ± 22.3 (33)
		GG	33.7 ± 6.8 (10)	3.3 ± 4.0 (17)	23.0 ± 17.7 (18)
LEP	−2548G>A	GG	30.7 ± 7.2 (18)	2.5 ± 2.6 (21)	31.5 ± 30.4 (16)
		GA	31.3 ± 7.7 (25)	2.4 ± 3.4 (34)	26.6 ± 20.2 (34)
		AA	33.2 ± 5.3 (17)	3.9 ± 3.9 (23)	21.4 ± 9.7 (20)
PYY	215G>C	GG	32.8 ± 8.5 (25)	3.2 ± 4.1 (32)	25.3 ± 12.4 (25)
	(Arg72Thr)	GC	30.7 ± 5.6 (27)	2.8 ± 3.1 (36)	24.6 ± 22.0 (27)
		CC	31.2 ± 5.0 (8)	1.8 ± 1.2 (10)	36.6 ± 34.5 (8)
NPY	204T>C	TT	28.5 ± 6.6 (19)	2.8 ± 3.7 (27)	33.6 ± 27.0 (21)
	(Ser68Ser)	TC	33.3 ± 7.7 (24)	2.6 ± 2.6 (31)	21.9 ± 18.7 (36)
		CC	32.8 ± 4.8 (17)	3.3 ± 4.1 (20)	26.3 ± 10.7 (13)
NPY2R	585T>C	TT	30.5 ± 6.4 (23)	2.4 ± 3.1 (28)	31.0 ± 21.2 (18)
	(Ile195Ile)	TC	33.4 ± 8.2 (24)	3.0 ± 3.5 (35)	25.9 ± 20.9 (34)
		CC	30.3 ± 4.1 (13)	3.3 ± 3.9 (15)	22.1 ± 20.8 (18)
CART	−156A>G	AA	32.4 ± 6.3 (16)	3.0 ± 4.1 (21)	23.7 ± 21.8 (16)
		AG	31.8 ± 8.2 (29)	3.0 ± 3.6 (40)	25.2 ± 18.5 (44)
		GG	30.5 ± 4.5 (15)	2.3 ± 1.8 (17)	34.7 ± 28.5 (10)

No significant differences in plasma PYY, GLP-1 and ghrelin levels were observed between genotypes. Data are presented as means ± s.d. (number of subjects).

postprandial response in plasma PYY levels in 477G>A in *GHSR* and for the 326A>G SNP in *LEPR*.

As age and BMI were not normally distributed among subjects, the Kruskal–Wallis and the Mann–Whitney *U*-tests were used to determine the associations between BMI and the SNPs and between the postprandial responses and BMI, respectively.

For the postprandial response in feelings of hunger and satiety, the number of subjects was sufficient to test for SNP–SNP interactions. To identify these interactions, the multifactor dimensionality reduction (MDR) method was used. MDR is a frequently used multi-locus method[26–28] that was applied as described earlier by Heidema *et al*.[29] Briefly, we applied the MDR software (http://www.epistasis.org) to our dataset using 10-fold cross-validation to determine the best model for main SNP–SNP effects. The 10-fold cross-validation was repeated 10 times, using a different seed value each time to protect against chance divisions of the dataset. Finally, applying the MDR permutation module, we tested the significance of the testing accuracy of the best model by forming 1000 datasets with the case status permuted randomly. This way, we validated for each phenotype whether the model was significantly associated with responder status. Subsequently, logistic regression was used for the significant models to obtain a statistical interpretation.[29]

As dietary restraint, disinhibition and perceived hunger were not normally distributed among subjects, the Kruskal–Wallis test was used to determine the association between these phenotypes and the relevant SNPs. Age, BMI, dietary restraint, disinhibition and perceived hunger were subsequently log-transformed for further analyses. Multiple linear regression analyses were used to test associations between dietary restraint, disinhibition and perceived hunger with the selected SNPs taking subject characteristics into account. *P*-values <0.05 were considered statistically significant.

Results

The genotypic and allelic distributions of the determined SNPs are provided in Table 1. All SNPs were in Hardy–Weinberg equilibrium except the 326A>G SNP (*LEPR*) ($\chi^2 = 4.2$; $P = 0.04$). Considering its borderline significance,

this SNP was included for further analyses. The 326A>G SNP (*LEPR*) and the 668A>G SNP (*LEPR*) were in linkage disequilibrium (LD) ($r=0.66$, $P<0.0001$), indicating that the information these SNPs provide overlaps. Both SNPs were included for further analyses.

For all SNPs, BMI was similar between genotypes ($P>0.05$). BMI was also similar between responders and non-responders for all postprandial responses ($P>0.05$). Correcting the associations for age and BMI did not change the results.

Table 3 Associations between postprandial responses and single nucleotide polymorphisms

Gene	*SNP*	*Response*	*Genotype*	*OR*	*95% CI*		P-*value*
GHRL	−501A>C	PYY_{ABS}	AC	3.27	1.01	10.62	0.05
			CC	10.29	1.74	60.90	0.01
GHSR	477G>A	PYY_{ABS}	GA+AA	0.33	0.11	0.97	0.04
LEPR	326A>G	$Ghrelin_{ABS}$	AG+GG	4.01	1.43	11.25	0.01
		$Ghrelin_{AUC}$	AG+GG	4.01	1.43	11.25	0.01
	668A>G	$Ghrelin_{AUC}$	GG	7.28	1.71	31.08	0.01
		$Hunger_{ABS}$	AG	0.34	0.13	0.88	0.03
		$Hunger_{AUC}$	AG	0.26	0.09	0.72	0.01
PYY	215G>C	$Ghrelin_{AUC}$	CC	—	—	—	0.01
LEP	−2548G>A	$Hunger_{RATE}$	GA	0.30	0.11	0.79	0.01

Response, dependent variable; genotype, independent variable; OR, odds ratio; 95% CI, 95% confidence interval for odds ratio; ABS, absolute postprandial response, i.e. the absolute difference between the baseline value and the minimal/maximal postprandial value; AUC, postprandial area under the curve; RATE, initial postprandial rate of response. All responses were dichotomized ('0'=non-responders; '1'=responders) and models were obtained using logistic regression. For all SNPs, the effects shown are relative to the subjects homozygous for the wild-type allele (reference group). For 477G>A and 326A>G, data were pooled for the least frequent allele. For 215G4C, subjects homozygous for the rare allele were not represented among the non-responders. Consequently, the *P*-value of the Fisher's exact test was used for this single nucleotide polymorphism (SNP).

Postprandial responses in plasma PYY, GLP-1 and ghrelin levels as well as feelings of hunger and satiety: single SNP associations

Baseline values for plasma PYY, GLP-1 and ghrelin levels, as well as for feelings of hunger and satiety, were similar between genotypes ($P>0.05$) (Table 2). The associations between the postprandial responses in plasma PYY, GLP-1 and ghrelin levels with the SNPs determined are described below and are shown in Table 3. Subjects with a high postprandial response in plasma PYY levels were over-represented among the CC and AC genotypes of the −501A>C SNP (*GHRL*) compared with the AA genotype ($P<0.05$). Subjects with a high postprandial response in plasma PYY levels were also more frequently homozygous for the common G allele of the 477G>A SNP (*GHSR*) than expected based on chance ($P<0.05$) (Table 3). No associations were found between the postprandial response in plasma GLP-1 levels and any of the SNPs determined.

Subjects with a high postprandial response in plasma ghrelin levels were over-represented among carriers of 109Arg-encoding allele (G) of the 326A>G SNP (*LEPR*) compared with subjects homozygous for the common 109Lys-encoding allele (A) ($P<0.01$). Furthermore, subjects with a high postprandial response in plasma ghrelin levels were over-represented among those homozygous for the 223Arg-encoding allele (G) of the 668A>G SNP (*LEPR*) compared with those homozygous for the 223Gln-encoding allele (A) ($P<0.01$). The postprandial response in plasma ghrelin levels was also associated with the 215G>C SNP (*PYY*); subjects with a high postprandial response were more frequently homozygous for the 72Thr-encoding allele (C)

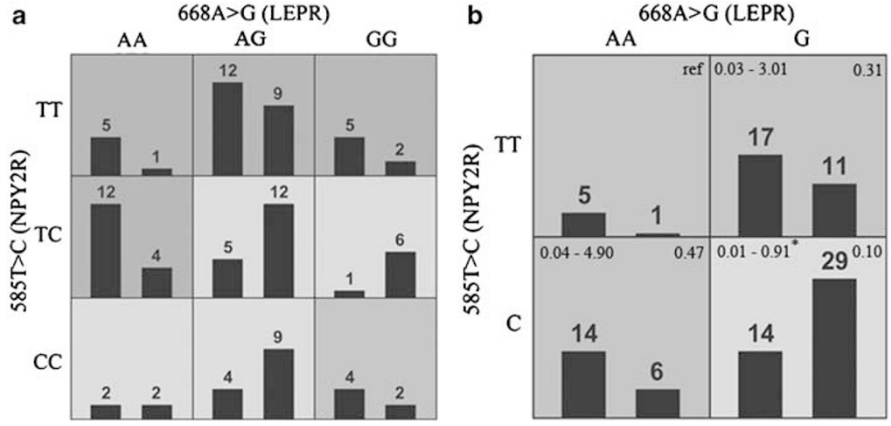

Figure 2 SNP–SNP interaction for the postprandial response in feelings of hunger (area under the curve) involving the 668A>G SNP (*LEPR*) and the 585T>C SNP (*NPY2R*). In each segment, the bar on the left represents the responders, that is, those with a high postprandial response in feelings of hunger, and the bar on the right represents the non-responders. (**a**) The multifactor dimensionality reduction (MDR) output obtained for this SNP–SNP interaction. (**b**) The statistical interpretation of the model obtained using logistic regression with AATT as the reference group. Carriers of the 223Arg-encoding allele (G) in 668A>G and carriers of the C allele in 585T>C were pooled (G and C, respectively). For each segment, the 95% confidence interval is shown in the left upper corner and the odds ratio in the right upper corner. *$P<0.05$.

than expected based on chance. As none of the subjects homozygous for the 72Thr-encoding allele was characterized by a low postprandial response in plasma ghrelin levels, it was not possible to obtain a parameter estimate for this association by logistic regression. Therefore, the Fisher's exact test was used instead ($P<0.01$). (Table 3).

Subjects with a high postprandial response in feelings of hunger were over-represented among subjects homozygous for the common G allele of the −2548G>A SNP (*LEP*) compared with heterozygous subjects ($P<0.05$) but not compared with subjects homozygous for the A allele. Similarly, subjects with a high postprandial response in feelings of hunger were over-represented among subjects homozygous for the common 223Gln-encoding allele of the 668A>G SNP (*LEPR*) compared with heterozygous subjects ($P<0.05$) but not compared with subjects homozygous for the 223Arg-encoding allele. No associations were observed between the postprandial response in feelings of satiety and any of the SNPs determined. An overview of all significant associations with odds ratios, 95% confidence intervals and *P*-values is provided in Table 3.

Postprandial responses in plasma PYY, GLP-1 and ghrelin levels as well as feelings of hunger and satiety: SNP–SNP interactions

MDR revealed a significant SNP–SNP interaction for the postprandial response in feelings of hunger involving the 668A>G SNP (*LEPR*) and the 585T>C SNP (*NPY2R*) (Figure 2a). The prediction accuracy of the model was 66.1% ($P=0.05$). Logistic regression subsequently showed that subjects with a high postprandial response in feelings of hunger were over-represented among subjects homozygous for the common allele in both SNPs compared with subjects carrying at least one 223Arg-encoding allele in 668A>G and at least one C allele in 585T>C ($P<0.05$) (Figure 2b).

Table 4 Associations between dietary restraint, disinhibition and perceived hunger with single nucleotide polymorphisms

Gene(s)	*SNP*	*Response*	*Parameter*	*B*	*95% CI*		*P-value*
GHSR	477G>A	Dietary restraint	AA	0.35	−0.04	0.73	0.08
			GA	0.30	0.03	0.57	0.03
			Gender	−0.36	−0.61	−0.11	0.006
			BMI	1.61	0.61	2.61	0.002
GHSR	477G>A	Disinhibition	AA	0.43	0.05	0.82	0.03
			GA	0.36	0.10	0.62	0.01
GHSR	477G>A	Perceived hunger	AA	0.54	0.08	1.00	0.02
			GA	0.33	0.03	0.64	0.03
NPY	204T>C		TC	0.44	0.11	0.78	0.01
			CC	0.42	0.02	0.83	0.04

Response, dependent variable; parameter, independent variable; B, unstandardized regression coefficient; 95% CI, 95% confidence interval for B. Models were obtained using linear regression analyses. For all single nucleotide polymorphisms (SNPs), the effects shown are relative to the subjects homozygous for the wild-type allele (reference group).

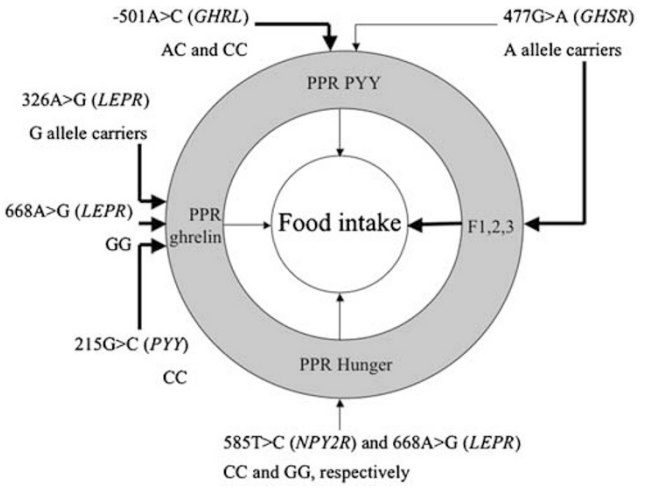

Figure 3 The association between the postprandial response (PPR) in plasma ghrelin and PYY levels and feelings of hunger, as well as cognitive aspects of food intake regulation (in gray) with SNPs encoding (an)orexigenic hormones and their receptors. F1,2,3 scores on dietary restraint, disinhibition of dietary restraint and perceived hunger as obtained using the three-factor eating questionnaire, respectively. Bold lines represent excitatory effects and thin lines represent inhibitory effects. The effects represent the genotypes shown compared with subjects homozygous for the common allele.

Dietary restraint, disinhibition and perceived hunger: single SNP associations

Values obtained for dietary restraint, disinhibition and perceived hunger were 5.1 ± 2.8, 4.5 ± 2.4 and 4.3 ± 2.7, respectively. Dietary restraint and disinhibition were higher in subjects with the AA and GA genotype of the 477G>A SNP (*GHSR*) than in subjects homozygous for the common G allele (5.6 ± 2.8 versus 4.2 ± 2.5 and 4.9 ± 2.4 versus 3.6 ± 2.2 for dietary restraint and disinhibition, respectively ($P<0.05$)). Perceived hunger was significantly associated with the 477G>A SNP (*GHSR*) and the 204T>C SNP (*NPY*) considered together, with perceived hunger being higher in subjects carrying the A allele in 477G>A and the C allele in 204T>C compared with subjects homozygous for the G and T alleles, respectively ($P<0.05$, $R^2=13\%$) (Table 4).

Dietary restraint was significantly associated with gender and BMI ($P<0.001$, $R^2=15\%$). Adding the 477G>A SNP (*GHSR*) to a model with gender and BMI significantly increased the explained variation to 20% ($P<0.05$), indicating an additive effect (Table 4). Disinhibition and perceived hunger were not associated with gender, age or BMI. As a result, correcting the associations for these variables did not change the results (Figure 3).

Discussion

Single SNP associations were observed between the postprandial response in plasma PYY levels and SNPs in *GHRL* and *GHSR* and between the postprandial response in plasma ghrelin levels and SNPs in *PYY* and *LEPR*. An SNP–SNP

interaction involving SNPs in *LEPR* and *NPY2R* was observed for the postprandial response in feelings of hunger. Dietary restraint and disinhibition were associated with an SNP in *GHSR*. Perceived hunger was associated with SNPs in *GHSR* and *NPY*.

C allele carriers of the −501A>C SNP (*GHRL*) were shown earlier to have higher fasting ghrelin levels than subjects with the AA genotype.[16] Increased plasma ghrelin levels are known to initiate individual meals[30] and increase food intake[31] in humans. On the basis of fasting plasma ghrelin levels, CC homozygous subjects are thus at increased risk for overeating. However, subjects homozygous for the C allele had a lower BMI and tended to have a lower waist circumference than the AC and AA genotypes,[16] suggesting an adequate food intake regulation. This paradox may be explained by an association observed in the present study; subjects with a high postprandial response in the anorectic hormone PYY were over-represented in subjects homozygous for the C allele compared with subjects homozygous for the common A allele. This association can be regarded as a long-term physiological adaptation to facilitate homeostasis in these subjects at increased risk for overeating as they are homozygous for the C allele in −501A>C.

The idea of genotype-related long-term physiological adaptations to facilitate homeostasis is supported by another observation. The 668A>G SNP (*LEPR*) was hypothesized earlier to affect the functionality of the LEPR, with the variant G allele (Arg) resulting in a leptin-resistant state.[14,32] Resistance to leptin is known to increase food intake, in extreme cases resulting in early-onset morbid obesity.[33,34] However, subjects homozygous for the 223Arg-encoding allele had a higher BMI than subjects homozygous for the common 223Gln-encoding allele in some,[14,35,36] but not all studies.[37,38] Again, long-term physiological adaptations required to facilitate homeostasis may be involved. First of all, subjects homozygous for the 223Arg-encoding allele were shown earlier to have higher plasma leptin levels.[14,35] Moreover, in the present study, subjects with a high postprandial response in plasma ghrelin levels were over-represented among subjects homozygous for the 223Arg-encoding allele compared with subjects homozygous for the 223Gln-encoding allele. Both effects protect subjects homozygous for the 223Arg-encoding allele from overeating, thereby facilitating homeostasis.

Analogous to the association shown for 668A>G, subjects with a higher postprandial response in plasma ghrelin levels were over-represented among carriers of the 109Arg-encoding allele of the 326A>G SNP (*LEPR*) compared with subjects homozygous for the common 109Lys-encoding allele. These SNPs were in LD, as was observed earlier by others.[35,37,39] This association reduces the risk for overeating in subjects homozygous for the 109Arg-encoding allele, consistent with results from Rosmond *et al*.[38] who showed a lower BMI and abdominal sagittal diameter in subjects with this genotype. This seems to confirm the association between the postprandial response in plasma ghrelin levels and the 326A>G SNP as a long-term physiological adaptation to facilitate homeostasis. However, in spite of their LD, no association was observed earlier between fasting plasma leptin levels and the 326A>G SNP.[14,35]

Two cases in which the postprandial responses in (an)orexigenic hormones actually reinforce direct effects were also identified in the present study. First of all, the A allele of the 477G>A SNP (*GHSR*) was shown earlier to increase the risk for obesity between 41 and 56%.[40] Baessler *et al*. concluded that the promoter regulatory elements of transcriptional initiation were probably affected. This way, genetic variation in *GHSR* alters the expression of the ghrelin receptor protein, which affects ghrelin signaling and ultimately the regulation of food intake. We showed that the increased risk for obesity in A allele carriers was reinforced by a reduced postprandial response in plasma PYY levels in subjects with this genotype. A cognitive component may also be involved, as A allele carriers of the 477G>A SNP had a higher dietary restraint, disinhibition and perceived hunger than subjects homozygous for the G allele. Restrained eaters were shown earlier to consume less energy, take fewer meals and show a higher preference for low-calorie foods than unrestrained eaters.[41,42] This suggests that subjects carrying the A allele may be at reduced risk for overeating. However, energy balance can only be maintained when a high score on dietary restraint is combined with a low score on disinhibition.[43] Furthermore, subjects suffering from feelings of hunger were previously concluded to be especially vulnerable to inhibit dietary restraint.[44] In subjects carrying the A allele, a high score on dietary restraint is accompanied by high scores on disinhibition and perceived hunger, thereby providing a cognitive explanation for an increased risk for overeating in these subjects, which is in line with the SNP's direct effect on ghrelin signaling.

Second, subjects homozygous for the common 72Arg-encoding allele in the 215G>C SNP (*PYY*) were shown earlier to have 20% lower fasting plasma PYY levels than subjects homozygous for the 72Thr-encoding allele.[45] Considering the role of PYY as an anorectic hormone, this is anticipated to increase the risk for overeating in subjects homozygous for the 72Arg-encoding allele. Indeed, these subjects had a higher risk for developing obesity than carriers of the 72Thr-encoding allele.[45] The present study showed that subjects with a higher postprandial response in plasma ghrelin levels were over-represented among subjects homozygous for the 72Thr-encoding allele compared with subjects homozygous for the common 72Arg-encoding allele. This provides subjects homozygous for the 72Thr allele with an additional protection against overeating compared with subjects homozygous for the 72Arg-encoding allele.

Subjects with a high postprandial response in feelings of hunger were over-represented among subjects homozygous for the common 223Gln-encoding allele in 668A>G (*LEPR*) compared with heterozygous subjects. However, subjects with a high postprandial response in feelings of hunger were equally represented among subjects homozygous for the

223Gln- and 223Arg-encoding alleles, making it difficult to explain this finding. A previously unidentified SNP–SNP interaction for the postprandial response in feelings of hunger, involving the 668A>G (*LEPR*) and 585T>C (*NPY2R*) SNPs provided more insight; subjects with a high postprandial response in feelings of hunger appeared to be over-represented in subjects homozygous for the common 223Gln-encoding allele (A) irrespective of the 585T>C SNP (Figure 2b). In contrast, in subjects carrying at least one 223Arg-encoding allele (G) subjects with a high postprandial response in feelings of hunger were over-represented in subjects homozygous for the common T allele in 585T>C compared with subjects carrying at least one C allele. As the 585T>C SNP is a silent transition, this SNP is likely to be in LD with a functional variant in the *NPY2R* locus.[46] To summarize, the 223Arg-encoding allele may increase food intake by decreasing leptin sensitivity, it may decrease food intake by increasing plasma leptin levels and by increasing the postprandial response in plasma ghrelin levels, and it may either increase or decrease food intake by influencing the postprandial response in feelings of hunger depending on the 585T>C SNP (*NPY2R*).

The phenotypes determined in the present study were not independent and two of the SNPs evaluated were in LD. Therefore, we did not correct for multiple testing, as correcting for multiple comparisons in a conventional way may discard true associations. Moreover, the study was explorative in nature: associations between the postprandial responses in (an)orexigenic hormones as well as feelings of hunger and satiety with genes encoding these proteins and their receptors have not been reported before.

In conclusion, we are the first to show that the postprandial responses in (an)orexigenic hormones and feelings of hunger to fixed meals providing an energy balance are associated with SNPs in genes encoding these proteins and their receptors. Indeed, part of the large inter-individual variability in these postprandial responses can thus be explained by genetic variation. Postprandial responses in (an)orexigenic hormones represent either long-term physiological adaptations to facilitate homeostasis or reinforce direct genetic effects. Finally, the present study shows that evaluating SNP–SNP interactions in addition to the conventional single SNP associations can elucidate findings that would otherwise remain inexplicable.

Acknowledgements

We thank Kristel Diepvens, Manuela Lejeune and Ananda Hochstenbach-Waelen for their contributions in acquiring the data on postprandial responses in (an)orexigenic hormones and feelings of hunger and satiety.

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