NIH Public Access
Author Manuscript
J Hypertens. Author manuscript; available in PMC 2014 January 01.

Published in final edited form as:
J Hypertens. 2013 January ; 31(1): 123–133. doi:10.1097/HJH.0b013e32835b053d.

Heredity and cardiometabolic risk: naturally occurring polymorphisms in the human neuropeptide Y_2 receptor promoter disrupt multiple transcriptional response motifs

Zhiyun Wei[a,b,c,e], **Kuixing Zhang**[a,b,c], **Gen Wen**[a,b,c], **Karthika Balasubramanian**[a,b,c], **Peian B. Shih**[a,b,c], **Fangwen Rao**[a,b,c], **Ryan S. Friese**[a,b,c], **Jose P. Miramontes-Gonzalez**[a,b,c], **Geert W. Schmid-Schoenbein**[a,b,c], **Hyung-Suk Kim**[d], **Sushil K. Mahata**[a,b,c], and **Daniel T. O'Connor**[a,b,c]

[a]Department of Medicine, Institute for Genomic Medicine, University of California at San Diego, the VA San Diego Healthcare System, La Jolla, California

[b]Department of Pharmacology, Institute for Genomic Medicine, University of California at San Diego, the VA San Diego Healthcare System, La Jolla, California

[c]Department of Bioengineering, Institute for Genomic Medicine, University of California at San Diego, the VA San Diego Healthcare System, La Jolla, California

[d]Department of Pathology, University of North Carolina at Chapel Hill, Chapel Hill, USA

[e]Bio-X Institutes, Shanghai Jiao Tong University, China

Abstract

Objectives—The neuropeptide Y_2 G-protein-coupled receptor (NPY2R) relays signals from PYY or neuropeptide Y toward satiety and control of body mass. Targeted ablation of the NPY2R locus in mice yields obesity, and studies of NPY2R promoter genetic variation in more than 10 000 human participants indicate its involvement in control of obesity and BMI. Here we searched for genetic variation across the human NPY2R locus and probed its functional effects, especially in the proximal promoter.

Methods and results—Twin pair studies indicated substantial heritability for multiple cardiometabolic traits, including BMI, SBP, DBP, and PYY, an endogenous agonist at *NPY2R*. Systematic polymorphism discovery by resequencing across *NPY2R* uncovered 21 genetic variants, 10 of which were common [minor allele frequency (MAF) >5%], creating one to two linkage disequilibrium blocks in multiple biogeographic ancestries. *In vivo, NPY2R* haplotypes were associated with both BMI (P=3.75E–04) and PYY (P=4.01E–06). Computational approaches revealed that proximal promoter variants G-1606A, C-599T, and A-224G disrupt predicted IRF1 (A>G), FOXI1 (T>C), and SNAI1 (A>G) response elements. In neuroendocrine cells transfected with *NPY2R* promoter/luciferase reporter plasmids, all three variants and their resulting haplotypes influenced transcription (G-1606A, P<2.97E–06; C-599T, P<1.17E–06; A-224G, P<2.04E–06), and transcription was differentially augmented or impaired by coexpression of either the cognate full-length transcription factors or their specific siRNAs at each site. Endogenous expression of transcripts for *NPY2R*, *IRF1*, and *SNAI1* was documented in neuroendocrine cells, and the *NPY2R* mRNA was differentially expressed in two neuroendocrine

© 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Correspondence to Daniel T. O'Connor, MD, Department of Medicine (0838), UCSD School of Medicine and VASDHS, 9500 Gilman Drive, La Jolla, CA 92093-0838, USA. Tel: +1 858 5340661; fax: +1 858 5340626; doconnor@ucsd.edu.

Conflicts of interest

The authors have no conflicts of interest to declare.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

tissues (adrenal gland, brainstem) of a rodent model of hypertension and the metabolic syndrome, the spontaneously hypertensive rat.

Conclusion—We conclude that common genetic variation in the proximal *NPY2R* promoter influences transcription factor binding so as to alter gene expression in neuroendocrine cells, and consequently cardiometabolic traits in humans. These results unveil a novel control point, whereby *cis*-acting genetic variation contributes to control of complex cardiometabolic traits, and point to new transcriptional strategies for intervention into neuropeptide actions and their cardiometabolic consequences.

Keywords

autonomic; genetics; hypertension; nervous system; obesity

INTRODUCTION

The neuropeptide Y (NPY) receptor Y_2 (NPY2R; OMIM 162642; IUPHAR Y_2) is a G-protein-coupled receptor responding to hormones peptide YY (PYY) [1] and NPY to control appetite and cardiovascular homeostasis. There are five subtypes of NPY receptor identified in mammals, four of which are functional in humans. Sub-types Y_1 and Y_5 have known roles in the stimulation of feeding, whereas Y_2 and Y_4 seem to have roles in appetite inhibition. NPY2R is widely expressed in tissues pertinent to cardiometabolic control, including the arcuate nucleus, a major integrator of appetite control in the hypothalamus. In previous studies, *NPY2R* genetic variants were associated with obesity or BMI in several populations, including whites [2,3], Asians [4], and Africans [5]. Indeed, studies of *NPY2R* promoter genetic variation in more than 10 000 individuals [2,3,6] indicate its involvement in control of obesity or BMI (on-line Table 1, http://links.lww.com/HJH/A209). NPY2R also cooperates with NPY in stress-induced obesity and the metabolic syndrome [7]. *NPY2R* genetic variants associate with such human cardiometabolic traits as high-density lipoprotein cholesterol [8], SBP [3], type 2 diabetes in men [9], and left ventricular hypertrophy [10]. Hypothalamus-targeted *NPY2R*-knockout mice showed a decrease in body weight despite an increase in food intake [11]. In the rat (http://rgd.mcw.edu), the *Npy2r* genetic locus underlies the confidence interval of a quantitative trait locus (QTL) for blood pressure (BP): BP QTL-90 (Bp90) [12]. Such diverse evidence indicates that *NPY2R* plays an indispensable role in the cardiometabolic syndrome.

In these studies, we first documented the role of heredity in cardiometabolic traits, using twin pair variance components, and then systematically searched for naturally occurring genetic variation across the human *NPY2R* locus. Because several of the discovered common variants occurred in a likely functional domain (the promoter), we probed their mechanistic consequences, beginning with bioinformatic motif analysis and proceeding to transfected promoter/luciferase reporter plasmids, site-directed mutagenesis, and characterization of *trans*-acting factors. We developed evidence that variation in the *NPY2R* promoter, especially at common variants G-1606A, C-599T, and A-224G, disrupt particular motifs (IRF1, FOXI1, and SNAI1 elements, respectively), creating differential *cis*-interactions and *trans*-interactions, to alter transcriptional activity and ultimately BP, body mass, and associated risk traits in the population.

PARTICIPANTS AND METHODS

Genomics

Systematic polymorphism discovery at the *NPY2R* locus—We studied the *NPY2R* locus in $n = 80$ participants ($2n = 160$ chromosomes) as described below under ‘Human participants’. Genomic DNA was prepared from leukocytes as described previously

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

[13]. Public draft human genome sequences were obtained from the University of California, Santa Cruz Genome Bioinformatics website (http://genome.ucsc.edu) and used as a scaffold for primer design. The base position numbers were according to the National Center for Biotechnology Information (NCBI) *NPY2R* source clone, RefSeq gene/transcript NM_000910.2. Promoter positions were numbered upstream of (−) the *NPY2R* exon-1 start (cap) site. PCR primers were designed by primer-3 [14] (http://frodo.wi.mit.edu/primer3/) to capture approximately 2000 bp of the proximal promoter, between approximately 500 bp to approximately2000 bp over each of the two exons (including 5′-UTR, 3′-UTR, and exon/intron borders), and regions highly conserved across species. Target regions were amplified and then dideoxy-sequenced using an ABI-3100 capillary sequencer (Applied Biosystems, Carlsbad, California, USA). Polymorphism (typically as heterozygosity) was visualized on the Applied Biosystems (ABI) tracings using Codon Code Aligner (http://www.codoncode.com/aligner).

Human participants

Resequencing the *NPY2R* locus—Human studies were approved by the University of California, San Diego (UCSD) Human Research Protection Program. Experiments were conducted with the understanding and consent of each participant. We studied the *NPY2R* locus in n =80 participants (2n =160 chromosomes) from four diverse biogeographic ancestry groups systematically sequenced for polymorphism discovery across the *NPY2R* locus: white (European ancestry, 2n =46 chromosomes), black (sub-Saharan African ancestry, 2n =50 chromosomes), Hispanic (Mexican American, 2n =32 chromosomes), and east Asian (2n =32 chromosomes).

UCSD twin pairs—Twin recruitment included access to a population birth record-based twin registry [15], as well as by newspaper advertisement, as described [16]. Description of the 362 participants in the twin heritability and allelic association studies has been published [17]. For human allelic and haplotype association studies, this twin group was expanded to 693 participants of European ancestry, derived from additional siblings from twinships and sibships, as previously described [18].

Statistics and informatics

Linkage disequilibrium and haplotypes—In the resequenced participants, patterns of linkage disequilibrium as well as haplotype frequencies were analyzed and visualized by the software Haploview (Broad Institute, Massachusetts, USA) [19]. Linkage disequilibrium blocks were derived by the confidence interval criterion and visualized by r^2 plot in Haploview from unphased diploid genotypes of n =80 resequenced participants (2n =160 chromosomes) from four diverse biogeographic ancestry groups systematically sequenced across the *NPY2R* locus. Common variants (minor allele frequency >5%) were used to establish linkage disequilibrium.

In the twins and siblings, haplotype-on-trait analyses were conducted by regression in R (reporting effect size as β, or slope per allele, as well as its SEM) with Haplo.glm in Haplo.stats [20] (http://mayoresearch.mayo.edu/schai-d_lab/software.cfm). Trait-associated haplotype GTT was present on 11.1 of chromosomes analyzed.

Bioinformatics: computational prediction of transcription factor-binding motifs overlying *NPY2R* promoter common variants—Multiple sequence alignments were performed by Clustal-W [21] (http://www.ebi.ac.uk/Tools/clustalw2/). Potential transcription factor binding motifs were predicted from the JASPAR [22] (http://jaspar.genereg.net/) and ConSite [23] (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/) databases.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Heritability and pleiotropy (shared genetic determination or genetic covariance, (ρ_G)—Estimates of heritability (h^2) ($h^2 = V_G/V_P$, wherein V_G is additive genetic variance and V_P is total phenotypic variance) were obtained using the twin-pair variance-component methodology implemented in the Sequential Oligogenic Linkage Analysis Routines (SOLAR) package [24] available at (http://txbiomed.org/departments/genetics/). This method maximizes the likelihood assuming a multivariate normal distribution of phenotypes in twin pairs (monozygotic versus dizygotic) with a mean dependent on a particular set of explanatory covariates. The null hypothesis (H_0) of no heritability is tested by comparing the full model, which assumes genetic variation (V_G), and a reduced model, which assumes no genetic variation, using a likelihood ratio test. Heritability estimates were adjusted for age and sex because of the effects of these covariates on several traits. Pleiotropy (genetic covariance for two correlated, heritable traits; i.e., the cross-product of trait heritabilities) [25] was estimated as the parameter ρ_G in SOLAR [25]. SOLAR also estimated the environmental covariance, as parameter ρ_E.

Functional studies of *NPY2R* genetic variation

Human phenotyping: peptide YY—Human PYY (total) was measured using a Linco (Millipore, St. Charles, Missouri, USA) HRP-TMB ELISA kit (catalog # EZHPYYT66K). EDTA-anticoagulated plasma was frozen and stored at −70°C prior to assay; this ELISA measures the enzyme by absorbance at 450 nm. The assay sensitivity is 1.4 pg/ml plasma, with an intra-assay coefficient of variability (CV) of 0.9–5.8%, and interassay CV of 3.7–16.5%. The assay equivalently recognizes PYY_{1-36} and PYY_{3-36}, but does not cross-react (at up to 50 nmol/l) with NPY, ghrelin, gastric inhibitory polypeptide, glucagon, glucagon-like peptide-1, leptin, insulin, C-peptide, amylin, or adiponectin. PYY distribution in human individuals was tested by the one-sample, two-tailed nonparametric Kolmogorov–Smirnov test in SPSS (IBM Corporation, New York, USA); untransformed PYY deviated from normality (P=0.002), whereas log[10]-transformed PYY did not display such deviation (P =0.291). Estimates of heritability (by variance components in SOLAR; see above) did not differ when performed on untransformed versus log[10]-transformed PYY data (see RESULTS).

Human single nucleotide polymorphism genotyping and marker-on-trait association—Single nucleotide polymorphism (SNP) genotypes at rs6851222 (Promoter G-1606A), rs6857715 (Promoter C-599T), and rs1047214 (Exon-2 T/C Ile195Ile) were chosen to span the *NPY2R* locus, and typed by the TaqMan method on an ABI-7900HT Fast Real-Time PCR System, with labeled probes synthesized at Applied Biosystems. Each SNP was in Hardy Weinberg equilibrium (all P>0.05). Haplotypes were derived from diploid genotype data, and haplotype-on-trait analyses were conducted by regression [20], or by Generalized Estimating Equations, with analyses adjusted for age, sex, and biogeographic ancestry.

***NPY2R* promoter haplotype/luciferase reporter design and construction—** Human *NPY2R* promoter fragments, corresponding to *NPY2R*-2323/+130 bp in *NPY2R* (NCBI *NPY2R* source clones: RefSeq gene/transcript NM_000910.2), were PCR-amplified from genomic DNA (after resequencing) and cloned into the polylinker (between KpnI and BglII sites) of the promoterless firefly luciferase reporter plasmid pGL3-Basic (Promega, Madison, Wisconsin, USA), as described [13]. Site-directed mutagenesis (QuikChange; Stratagene, Santa Clara, California, USA) created the required variant at position −1606, −599, and −224 (Supplemental Digital Content Fig. S1, http://links.lww.com/HJH/A209). Super-coiled plasmids were purified using NucleoBond Xtra Maxi kits (740414.10; Machery-Nagel, Bethlehem, Pennsylvania, USA) prior to transfection, and verified by

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

sequencing. Promoter positions are numbered upstream (−) of the transcriptional start (cap) site.

Luciferase reporter assays of *NPY2R* promoter variants—PC12 rat pheochromocytoma cells were transfected (at 60–80% confluence, 1 day after 1 : 4 splitting in 24-well plate) with 500 ng of supercoiled promoter/firefly luciferase reporter plasmid per well, by the liposome method (Transfectin; Bio-Rad, Hercules, California, USA). The firefly luciferase activity in cell lysates was measured 24 h after transfection, using the luciferase assay system (Promega), and the results were expressed as the ratio of firefly activity/total protein in the lysate, as described [13]. Each experiment included at least three replicates. Results were expressed as mean ± SEM. Statistical significance was calculated using Student's *t*-test or ANOVA, and significance was established at the *P* value less than 0.05 level. Inspection of the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) indicates that transcripts for *NPY2R* are abundantly expressed in the adrenal gland (GEO dataset GDS3556 and GDS2374) as well as PC12 chromaffin cells (GDS2555).

Exogenous/cotransfected transcription factors—Eukaryotic expression plasmids containing cDNAs encoding transcription factors IRF1 (rat; clone ID 7099391), FOXI1 (human; clone ID 5185923) and SNAI1 (human; clone ID 4537122) were from Open Biosystems (Huntsville, Alabama, USA). cDNAs were obtained in either pExpress-1 or cytomegalovirus promoter (pCMV)-SPORT6 plasmids, and subcloned if needed into a eukaryotic pCMV expression vector (pcDNA-3.1). One hundred nanograms of each transcription factor expression plasmid, or 100 ng pcDNA-3.1 empty vector (control), was cotransfected into PC12 cells, along with 500 ng of *NPY2R* promoter/luciferase reporter, wild-type versus variants. After 24 h, cells were lysed and luciferase activities were assayed as described above and normalized by total protein. Response of the *NPY2R* promoter to exogenous transcription factor was revealed by comparison of the normalized luciferase activity between the transcription factor-transfected group and the mock-transfected (empty vector, pcDNA 3.1) group.

Exogenous/cotransfected siRNAs—Silencer select predesigned siRNAs targeting IRF1 (rat; siRNA ID s127967), FOXI1 (rat; siRNA ID s220491), or SNAI1 (rat; siRNA ID s137986) were from Ambion (Applied Biosystems). Silencer select negative control #1 siRNA (part number 4390843) was used as the negative control. Six nanomoles per litre final concentration of each transcription factor siRNA, or negative control siRNA, was cotransfected into PC12 cells, along with 500 ng of *NPY2R* promoter/luciferase reporter wild-type versus variant. After 24 h, cells were lysed and luciferase activities were assayed as described above and normalized by total protein. Response of the *NPY2R* promoter to exogenous siRNAs was revealed by comparison of the normalized luciferase activity between the transcription factor siRNA-transfected group and the mock-transfected (negative control siRNA) group.

Quantification of endogenous transcripts by real-time PCR: *NPY2R* itself and transcripts for factors whose binding motifs are disrupted by *NPY2R* promoter variants (*IRF1, FOXI1, SNAI1*)—Total RNA was extracted from cells (neuroendocrine PC12) or organs under each experimental state, using an ABI 6700 automated nucleic acid workstation, and quantitative real-time PCR (RT-PCR) was performed on mRNA→cDNA with the ABI-7700 TaqMan platform, using fluorescent reporter-tagged oligonucleotide primers, and normalization of data to β-actin expression in the same sample. Threshold cycle (C_t) is determined for both the specific target mRNA/cDNA as well as β-actin, and the difference in C_t (for target mRNA versus β-actin mRNA)

NIH-PA Author Manuscript

is normalized to the average for that state (e.g., control versus experimental), by the $\Delta\Delta C_t$ method [26].

Experimental animals: spontaneously hypertensive rat and Wistar–Kyoto rat—Animal studies were performed with age-matched, adult (12–17 weeks) male spontaneously hypertensive rat (SHR) and Wistar–Kyoto (WKY) rat strains from Charles River Laboratories (Wilmington, Massachusetts, USA). Features of the Charles River colonies, including BP monitoring, are given at (http://www.criver.com/EN-US/PRODSERV/BYTYPE/RESMODOVER/RESMOD/Pages/SHRRat.aspx). Isoflurane was used for terminal anesthesia of SHR and WKY rats. Adrenal glands and brainstem were isolated from each rat (n =9 per group), immediately frozen in liquid nitrogen, and then stored at −80°C prior to RNA extraction and RT-PCR. Rats were studied according to a protocol approved by the Animal Subjects Committee of the University of California at San Diego, and research was conducted in accordance with institutional guidelines.

RESULTS

Heredity, pleiotropy, and cardiometabolic traits in humans

Twin pair variance component analyses indicate that multiple cardiometabolic traits display substantial and significant (P=0.0001) heritability (h^2) (Fig. 1a), including BMI (h^2 =86 ± 2%), SBP (h^2 =46 ± 6%), DBP (h^2 =52 ± 6%), and circulating PYY (h^2 =51 ± 6%), the principal endogenous ligand for the NPY2R. Heritability estimates for BMI, SBP, and DBP were consistent with previously reported values [17]. Using the twin method, we also investigated genetic pleiotropy (shared genetic determination or genetic covariance) between BMI and other cardiometabolic traits (Fig. 1b). BMI displayed significant genetic covariance with SBP (P=9.31E–05), DBP (P=7.74E–04), and PYY (P=3.0E–02); by contrast, environmental covariance (or shared environmental determination, ρ_E) was not significant for these same traits.

Polymorphism discovery across *NPY2R*

Located on chromosome 4q31, *NPY2R* spans two exons (one coding) with one intervening sequence (intron). We resequenced approximately 1800 bp of proximal promoter, each of exon-1 and exon-2 [down to the first polyadenylation site (bold): 5′-TACTA**AATAAA**ACAAT-3′], and adjacent intron/exon borders (Fig. 2) in 2n =160 chromosomes derived from four biogeographic ancestry groups (Table 1). We identified 21 variants (18 SNPs, 3Ins/Del) in these individuals. Of these variants, 10 are common [minor allele frequency (MAF) >5%], including two in the open reading frame within coding exon-2 (both synonymous), T +5895C (Ile195Ile) and T +6242C (Ile312Ile), whereas the rest are located in the proximal promoter.

Biogeographic ancestry and *NPY2R* linkage disequilibrium

NPY2R common allele frequencies did not differ across the four biogeographic ancestry groups (Table 1). To visualize patterns of marker-on-marker association, pair-wise linkage disequilibrium correlations among the eight common (MAF >5%) SNPs were quantified by the confidence interval method across the *NPY2R* locus. In each biogeographic ancestry group, two blocks of linkage disequilibrium were maintained, with one in the promoter region (Fig. 3a).

Neuropeptide Y2 receptor haplotype effects on traits

We ‘tagged’ the human *NPY2R* gene with three SNPs spanning the locus (Fig. 3b): haplotype GTT (found on 11.1% of chromosomes) was associated significantly with both

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

BMI (*P* =3.75E–04) and PYY secretion (*P* =4.01E–06), and the principal effect accrued to GTT homozygotes (with two copies of that haplotype per diploid genome); the GTT effect size (or slope) was positive for BMI (1.93 ± 0.48 kg/m^2 per copy), although negative for PYY (–26.3 ±5.65 pg/ml per copy). Perhaps these pleiotropic effects of haplotype GTT involve increased response to PYY, with consequent fall in this anorexigenic hormone and ultimately an increase in BMI.

Endogenous *NPY2R* mRNA expression in a disease model in rodents: spontaneously hypertensive rat/Wistar–Kyoto rat

NPY2R mRNA expression was increased significantly in two key neuroendocrine tissues of the SHR (Fig. 4a): both the adrenal gland (by ~2.6-fold, *P* =0.002) and the brainstem (by ~1.5-fold, *P* =0.027).

Genetic variation in the proximal human *NPY2R* promoter: consensus motifs

Core promoter: nonpolymorphic motifs—Motifs identified did not include a consensus TATA box near the transcriptional start site; the closest partial TATA (i.e., T/A-rich) match on the (+) strand was 5′-(–113 bp)-AAAcTT-(–108 bp)-3′, whereas the nearest potential CAAT box was on the (–) strand at 5′ (–420 bp)-CCAAT (–424 bp)-3′. There was no proximal cAMP response element. The 13 G/C-rich (consecutive G/C 6 bp) regions were noted in the proximal promoter, as were 4 E-boxes (CANNTG). One of the G/C-rich domains constituted a consensus match for a B recognition element [27], on the very proximal (+) strand at 5′-(–49 bp) GGGCGCC (–43 bp)-3′. The closest potential initiator (Inr) elements [28] (consensus 5′-YYA$_{+1}$NWYY-3′) were located at 5′-(–244 bp)CCAGTCC(–238 bp)-3′ (+ strand) and 5′-(+151 bp)TTACACT(+145 bp)-3′ (– strand). None of these core elements were polymorphic across 2*n* =160 human chromosomes.

Polymorphisms—We identified 16 polymorphisms in the promoter (Table 1), eight of which were common (MAF >5%). Of note, the very proximal 'core' promoter (–186/+85 bp) was devoid of common variation. At promoter variants G-1606A, C-599T, and A-224G, we identified motifs likely to be disrupted by the sequence change (see below).

***NPY2R* promoter haplotypes affect gene expression—**Constructed from three common SNPs (G-1606A, C-599T and A-224G) that were predicted to be functional (see below), eight haplotypes were created by site-directed mutagenesis from the most common promoter haplotype (alleles: G-1606, C-599 and A-224; 55.4% of chromosomes in our sample). *NPY2R* promoter/luciferase reporters with various haplotypes had significantly different expression activities (one-way ANOVA: *P* =1.12E–23; Fig. 5a). We used two-way ANOVA to probe individual SNP effects on gene expression: each individual SNP, as well as their binary and ternary interactions, displayed significant influences on reporter expression (*P* =5.00E–06; Fig. 5b).

Neuropeptide Y2 receptor G-1606A polymorphism: role of an *IRF1* activator-binding site

Sequence conservation/alignment—G-1606A is located in a region highly conserved across sequenced primates (Fig. 4a), with the G allele ancestral in the human lineage, as judged by the chimp sequence (Fig. 6a). In this conserved local region, there is a partial consensus match for an IRF1 site (VAAARYG**A**AASY; –1606 in bold) with an improved match for the A allele (10/12 bp match) over the G allele (9/12 bp match) (Fig. 4a).

Exogenous *IRF1* transcription factor: increased *NPY2R* promoter-driven reporter expression, A>G allele—During *NPY2R* promoter/luciferase reporter transfection/expression into chromaffin cells (cotransfection with empty vector pcDNA 3.1;

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Fig. 6b), the A allele displayed greater expression than the G allele (A>G). Cotransfection/expression of the IRF1 transcription factor increased reporter expression and amplified the difference in expression between the two alleles (Fig. 4b, *P*=0.001).

Exogenous *IRF1* siRNA: decreased *NPY2R* promoter-driven reporter expression, A>G allele—During *NPY2R* promoter/luciferase reporter cotransfection with negative control siRNA into chromaffin cells (Fig. 6c), the A allele once again displayed greater expression than the G allele (A>G). Cotransfection of *IRF1* siRNA decreased reporter expression and attenuated the difference of expression between the two alleles (Fig. 6C, *P*=8.72E–06).

Neuropeptide Y2 receptor C-599T polymorphism: role of an activator *FOXI1* binding site

Sequence conservation/alignment—C-599T is located in a region highly conserved across sequenced primates (Fig. 7a), with the T allele ancestral in the human lineage, as judged by the chimp sequence (Fig. 7a). In this conserved local region, there is a total consensus match for a *FOXI1* site (**T**RTTTRKWD; –599 in **bold**) with an improved match for the T allele (9/9 bp match) over the C allele (8/9 bp match) (Fig. 7a).

Exogenous *FOXI1* transcription factor: increase in *NPY2R* promoter-driven reporter expression, T>C allele—During *NPY2R* promoter/luciferase reporter transfection into chromaffin cells (cotransfection with empty vector pcDNA 3.1; Fig. 7b), the T allele displayed greater expression than the C allele (T>C). Cotransfection/expression of FOXI1 transcription factor increased reporter expression and amplified the difference of expression between the two alleles (Fig. 7b, *P*=5.57E–06).

Exogenous *FOXI1* siRNA: decrease in *NPY2R* promoter-driven reporter expression, T>C allele—During *NPY2R* promoter/luciferase reporter, cotransfection with negative control siRNA into chromaffin cells (Fig. 7c), the T allele displayed greater expression than the C allele (T>C). Cotransfection of FOXI1 siRNA decreased reporter expression and attenuated the difference of expression between two alleles (Fig. 7c, *P* =0.010).

Neuropeptide Y2 receptor A-224G polymorphism: role of a *SNAI1* repressor binding site

Sequence conservation/alignment—A-224G is located in a region highly conserved across sequenced primates (Fig. 8a), with the A allele ancestral in the human lineage, as judged by the chimp sequence (Fig. 8a). In this conserved local region, there is a partial consensus match for an SNAI1 site (C**A**GGTG; –224 in bold) with an improved match for the A allele (5/6 bp match) over the G allele (4/6 bp match) (Fig. 8a).

Exogenous *SNAI1* transcription factor: decrease in *NPY2R* promoter-driven reporter expression, A>G allele—During *NPY2R* promoter/luciferase reporter transfection into chromaffin cells (cotransfection with empty vector pcDNA 3.1; Fig. 8b), the G allele displayed greater expression than the A allele (G>A). Cotransfection of the SNAI1 transcription factor decreased reporter expression and amplified the difference of expression between the two alleles (Fig. 8b, *P*=0.034).

Exogenous *SNAI1* siRNA: increase in *NPY2R* promoter-driven reporter expression, A>G allele—During *NPY2R* promoter/luciferase reporter cotransfection with negative control siRNA into chromaffin cells (Fig. 8c), the G allele displayed greater expression than the A allele (G>A). Cotransfection of SNAI1 siRNA increased reporter

expression and attenuated the difference of expression between two alleles (Fig. 8c, *P* =0.019).

Endogenous mRNA expression in neuroendocrine cells: *NPY2R* and transcription factors whose binding is disrupted by *NPY2R* promoter common genetic variation (*IRF1, FOXI1, SNAI1*)

We used PC12 (rat pheochromocytoma) cells as an experimental system to test the effects of potentially allele-specific transcription factors, but are the receptor and these transcription factors endogenously expressed in this model system (Fig. 4b)? *NPY2R* itself, as well as the transcription factors *IRF1* and *SNAI1*, displayed substantial expression in PC12 cells, whereas *FOXI1* expression was undetectable.

DISCUSSION

Overview

NPY2R represents a central control point for the PYY/NPY regulatory pathway. In this study, we explored whether and how common genetic variations in the *NPY2R* promoter affect gene expression. We present evidence from several approaches (genomic, bioinformatic, transfection, *trans*-activation, and siRNA inhibition) in which we found that promoter variants G-1606A, C-599T, and A-224G conferred functional changes onto *NPY2R* expression, and that particular transcription factors were implicated. We, thus, present evidence of previously unexpected *cis*-variation in the regulation of *NPY2R* expression.

Cardiometabolic traits and *NPY2R* genetic variation

We found that multiple cardiometabolic traits are highly heritable, and also display shared genetic determination (Fig. 1). Associations between *NPY2R* SNPs and obesity are widely investigated in multiple populations, with substantial agreement that significant marker-on-trait effects occur [29]. We too could replicate such effects, in that a haplotype across the *NPY2R* locus influenced both BMI and PYY (Fig. 3b). Thus, in this report we describe a potential genetic contributor to dysregulation of body mass: genetic variation at the *NPY2R* locus (Figs 2 and 3).

Neuropeptide Y2 receptor promoter variants G-1606A, C-599T, and A-224G

We focused on three promoter polymorphisms that are not only common (high MAF) but also predicted to influence transcription factor binding, by bioinformatic analyses. On the basis of this strategy, the G-1606A, C-599T, and A-224G were advanced to further investigation. Frequencies of their promoter haplotypes are shown in Fig. 5.

Of note for the physiological significance of these results, we detected abundant transcripts in neuroendocrine PC12 cells (Fig. 4b) for *NPY2R* itself, *IRF1*, and *SNAI1*. In addition, query of the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) indicates that transcripts for *NPY2R, IRF1* (binding G-1606A), *FOXI1* (binding C-599T), and *SNAI1* (binding A-224G) are expressed endogenously in PC12 chromaffin cells, by inspection of the following GEO transcript datasets: GDS3436 [30], GDS1038–1039 [31], and GDS2555 [32].

Results in context with the literature

Common genetic variation in the *NPY2R* promoter [2,3,6] has been associated with obesity or BMI traits in studies of over 10 000 individuals (on-line Table 1, http://links.lww.com/HJH/A209); in one case [6], the effect size (as Cramer's phi) suggested that *NPY2R*

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

promoter genetic variation might account for up to approximately 9.3% of trait variance in the population. Among the three promoter variants evaluated in depth in our studies, C-599T (rs6857715) was implicated in one of these association studies: C-599T was associated with both adult and childhood obesity in a French sample [6], and this variant also had an effect on high-density lipoprotein cholesterol [8]; C-599T was a component of functional promoter haplo-types on gene expression (Figs 5 and 7) as well as the BMI/PYY-associated GTT haplotype in our population (Fig. 3b).

Furthermore, each of the three transcription factors (Figs 6–8) whose binding is altered by *NPY2R* promoter variants is already implicated in cardiometabolic function. A meta-analysis of genome-wide association studies revealed the influence of IRF1 on circulating C-reactive protein level, which is strongly associated with cardiovascular disease [33]. IRF1 also plays a key role in development of insulitis and diabetes in a mouse model [34]. FOXI1 may be necessary for expression of at least four subunits and proper assembly of the vacuolar H^+-ATPase complex [35], whose activity has an impact on hypertension [36]. SNAI1 transcriptionally controls cardiovascular progenitor cell formation through epicardial epithelial-mesenchymal transition [37], and such function is regulated by glucose metabolism [38].

Limitations of this study

A number of issues remain unexplored by our studies. For effects in very large sample sizes (>10 000 participants; on-line Table 1, http://links.lww.com/HJH/A209), we rely on the findings of other groups [2,3,6] that *NPY2R* promoter polymorphism influences obesity, especially for C-599T [6], although we did find evidence for such effects in our own population (Fig. 3b). Second, the *cis*-interactions*/trans*-interactions that we observed in transfected cells (Figs 6–8) are novel, and thus not yet established *in vivo*, although we did find evidence of differential expression of *NPY2R* in neuroendocrine tissues of the SHR (Fig. 4a), as well as endogenous expression of the pertinent transcripts in neuroendocrine cells (Fig. 4b).

Conclusions and perspectives

We conclude that cardiometabolic traits are highly heritable, that NPY genetic variation influences such traits (including BMI and PYY), and that within the *NPY2R* promoter, common polymorphisms are associated with alterations in transcriptional efficiency. The functional effects of polymorphism seem to arise from differential actions of specific transcription factors at the *NPY2R* promoter: IRF1 functioning as an activator disrupted by G-1606A bi-allelic variation, FOXI1 acting as an activator disrupted by C-599T, and SNAI1 acting as a repressor disrupted by A-224G. The results raise the potential for novel alterations in *cis*-interactions for control of PYY responses, thus, augmenting our understanding of molecular events underlying interindividual variation in energy balance, and the genetic predisposition toward obesity, a potent risk factor for cardiovascular disease.

Acknowledgments

Sources of funding are National Institutes of Health [HL58120; 1UL1RR031980 (UCSD Clinical and Translational Research Institute); MD000220 (UCSD Comprehensive Research Center in Health Disparities, CRCHD)], Department of Veterans Affairs.

Abbreviations

NPY	neuropeptide Y

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NPY2R	neuropeptide Y2 receptor
PYY	peptide YY
SNP	single nucleotide polymorphism

References

1. Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. Nature. 2002; 418:650–654. [PubMed: 12167864]
2. Torekov SS, Larsen LH, Andersen G, Albrechtsen A, Glümer C, Borch-Johnsen K, et al. Variants in the 5′ region of the neuropeptide Y receptor Y2 gene (NPY2R) are associated with obesity in 5,971 white subjects. Diabetologia. 2006; 49:2653–2658. [PubMed: 17019604]
3. Lavebratt C, Alpman A, Persson B, Arner P, Hoffstedt J. Common neuropeptide Y2 receptor gene variant is protective against obesity among Swedish men. Int J Obes (Lond). 2006; 30:453–459. [PubMed: 16331299]
4. Zhang J, Wang HJ, Ma J. Association between obesity and the polymorphism of neuropeptide Y2 receptor gene in children and adolescents. Zhonghua Liu Xing Bing Xue Za Zhi. 2009; 30:695–698. [PubMed: 19957593]
5. Friedlander Y, Li G, Fornage M, Williams OD, Lewis CE, Schreiner P, et al. Candidate molecular pathway genes related to appetite regulatory neural network, adipocyte homeostasis and obesity: results from the CARDIA Study. Ann Hum Genet. 2010; 74:387–398. [PubMed: 20642810]
6. Siddiq A, Gueorguiev M, Samson C, Hercberg S, Heude B, Levy-Marchal C, et al. Single nucleotide polymorphisms in the neuropeptide Y2 receptor (NPY2R) gene and association with severe obesity in French white subjects. Diabetologia. 2007; 50:574–584. [PubMed: 17235527]
7. Kuo LE, Kitlinska JB, Tilan JU, Li L, Baker SB, Johnson MD, et al. Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. Nat Med. 2007; 13:803–811. [PubMed: 17603492]
8. Takiguchi E, Fukano C, Kimura Y, Tanaka M, Tanida K, Kaji H. Variation in the 5′-flanking region of the neuropeptide Y2 receptor gene and metabolic parameters. Metabolism. 2010; 59:1591–1596. [PubMed: 20359722]
9. Campbell CD, Lyon HN, Nemesh J, Drake JA, Tuomi T, Gaudet D, et al. Association studies of BMI and type 2 diabetes in the neuropeptide y pathway: a possible role for NPY2R as a candidate gene for type 2 diabetes in men. Diabetes. 2007; 56:1460–1467. [PubMed: 17325259]
10. Arnett DK, Devereux RB, Rao DC, Li N, Tang W, Kraemer R, et al. Novel genetic variants contributing to left ventricular hypertrophy: the Hyper-GEN study. J Hypertens. 2009; 27:1585–1593. [PubMed: 19593212]
11. Sainsbury A, Schwarzer C, Couzens M, Fetissov S, Furtinger S, Jenkins A, et al. Important role of hypothalamic Y2 receptors in body weight regulation revealed in conditional knockout mice. Proc Natl Acad Sci U S A. 2002; 99:8938–8943. [PubMed: 12072562]
12. Garrett MR, Rapp JP. Multiple blood pressure QTL on rat chromosome 2 defined by congenic Dahl rats. Mamm Genome. 2002; 13:41–44. [PubMed: 11773968]
13. Wen G, Mahata SK, Cadman P, Mahata M, Ghosh S, Mahapatra NR, et al. Both rare and common polymorphisms contribute functional variation at CHGA, a regulator of catecholamine physiology. Am J Hum Genet. 2004; 74:197–207. [PubMed: 14740315]
14. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Method Mol Biol. 2000; 132:365–386.
15. Cockburn M, Hamilton A, Zadnick J, Cozen W, Mack TM. The occurrence of chronic disease and other conditions in a large population-based cohort of native Californian twins. Twin Res. 2002; 5:460–467. [PubMed: 12537877]
16. Zhang L, Rao F, Wessel J, Kennedy BP, Rana BK, Taupenot L, et al. Functional allelic heterogeneity and pleiotropy of a repeat polymorphism in tyrosine hydroxylase: prediction of catecholamines and response to stress in twins. Physiol Genomics. 2004; 19:277–291. [PubMed: 15367723]

NIH-PA Author Manuscript

17. Wessel J, Moratorio G, Rao F, Mahata M, Zhang L, Greene W, et al. C-reactive protein, an 'intermediate phenotype' for inflammation: human twin studies reveal heritability, association with blood pressure and the metabolic syndrome, and the influence of common polymorphism at catecholaminergic/beta-adrenergic pathway loci. J Hypertens. 2007; 25:329–343. [PubMed: 17211240]
18. Shih PA, Wang L, Chiron S, Wen G, Nievergelt C, Mahata M, et al. Peptide YY (PYY) gene polymorphisms in the 3'-untranslated and proximal promoter regions regulate cellular gene expression and PYY secretion and metabolic syndrome traits in vivo. J Clin Endocrinol Metab. 2009; 94:4557–4566. [PubMed: 19820027]
19. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005; 21:263–265. [PubMed: 15297300]
20. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. Am J Hum Genet. 2002; 70:425–434. [PubMed: 11791212]
21. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994; 22:4673–4680. [PubMed: 7984417]
22. Wasserman WW, Sandelin A. Applied bioinformatics for the identification of regulatory elements. Nat Rev Genet. 2004; 5:276–287. [PubMed: 15131651]
23. Sandelin A, Wasserman WW, Lenhard B. ConSite: Web-based prediction of regulatory elements using cross-species comparison. Nucleic Acids Res. 2004; 32(suppl 2):W249–W252. [PubMed: 15215389]
24. Almasy L, Blangero J. Multipoint quantitative-trait linkage analysis in general pedigrees. Am J Hum Genet. 1998; 62:1198–1211. [PubMed: 9545414]
25. Falconer, DS.; Mackay, TFC. Introduction to quantitative genetics. 4. Harlow, Essex, UK: Longman; 1996.
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]
27. Lagrange T, Kapanidis AN, Tang H, Reinberg D, Ebright RH. New core promoter element in RNA polymerase II-dependent transcription: sequence-specific DNA binding by transcription factor IIB. Genes Dev. 1998; 12:34–44. [PubMed: 9420329]
28. Javahery R, Khachi A, Lo K, Zenzie-Gregory B, Smale ST. DNA sequence requirements for transcriptional initiator activity in mammalian cells. Mol Cell Biol. 1994; 14:116–127. [PubMed: 8264580]
29. Naveilhan P, Hassani H, Canals JM, Ekstrand AJ, Larefalk A, Chhajlani V, et al. Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor. Nat Med. 1999; 5:1188–1193. [PubMed: 10502824]
30. Yamada M, Shida Y, Takahashi K, Tanioka T, Nakano Y, Tobe T. Prg1 is regulated by the basic helix-loop-helix transcription factor Math2. J Neurochem. 2008; 106:2375–2384. [PubMed: 18643870]
31. Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM, et al. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell. 2004; 119:1041–1054. [PubMed: 15620361]
32. Lattanzi W, Bernardini C, Gangitano C, Michetti F. Hypoxia-like transcriptional activation in TMT-induced degeneration: microarray expression analysis on PC12 cells. J Neurochem. 2007; 100:1688–1702. [PubMed: 17348866]
33. Dehghan A, Dupuis J, Barbalic M, Bis JC, Eiriksdottir G, Lu C, et al. Meta-analysis of genome-wide association studies in >80 000 subjects identifies multiple loci for C-reactive protein levels. Circulation. 2011; 123:731–738. [PubMed: 21300955]
34. Nakazawa T, Satoh J, Takahashi K, Sakata Y, Ikehata F, Takizawa Y, et al. Complete suppression of insulitis and diabetes in NOD mice lacking interferon regulatory factor-1. J Autoimmun. 2001; 17:119–125. [PubMed: 11591120]

35. Vidarsson H, Westergren R, Heglind M, Blomqvist SR, Breton S, Ener-back S. The forkhead transcription factor Foxi1 is a master regulator of vacuolar H-ATPase proton pump subunits in the inner ear, kidney and epididymis. PLoS ONE. 2009; 4:e4471. [PubMed: 19214237]
36. Wei Z, Biswas N, Wang L, Courel M, Zhang K, Soler-Jover A, et al. A Common Genetic Variant in the 3′-UTR of Vacuolar H+-ATPase ATP6V0A1 Creates a Micro-RNA Motif to Alter Chromogranin A (CHGA) Processing and Hypertension Risk. Circ Cardiovasc Genet. 2011; 4:381–389. [PubMed: 21558123]
37. Martínez-Estrada OM, Lettice LA, Essafi A, Guadix JA, Slight J, Velecela V, et al. Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. Nat Genet. 2010; 42:89–93. [PubMed: 20023660]
38. Park SY, Kim HS, Kim NH, Ji S, Cha SY, Kang JG, et al. Snail1 is stabilized by O-GlcNAc modification in hyperglycaemic condition. EMBO J. 2010; 29:3787–3796. [PubMed: 20959806]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

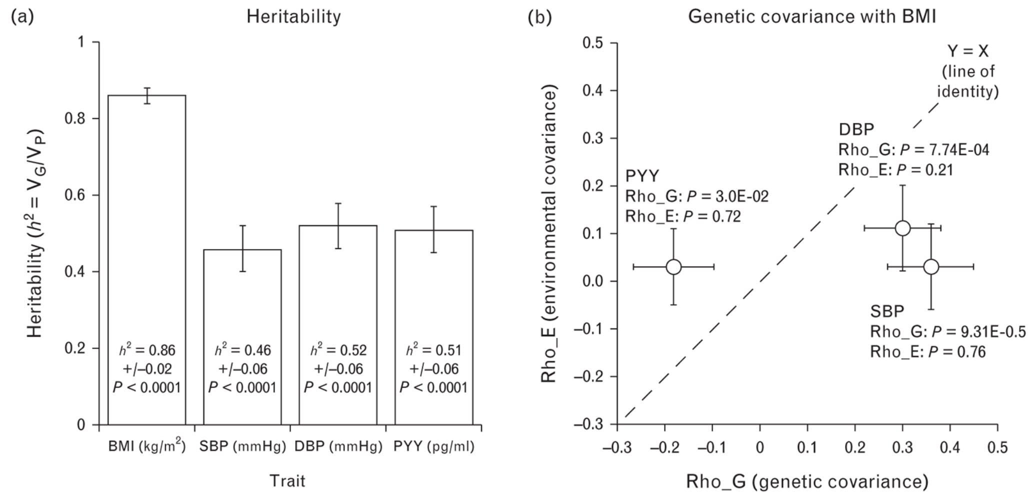

FIGURE 1.

Heredity, pleiotropy, and human cardiometabolic traits. (a) Heritability (h^2): twin pair variance components. h^2 is the fraction of trait variance accounted for by genetic variance (i.e., $h^2 = V_G / V_P$). h^2 (±SEM, with significance for h^2) is displayed for BMI, SBP, DBP, and circulating plasma peptide YY (PYY) concentration. (b) Pleiotropy: shared genetic determination, or genetic covariance (ρG) for BMI with other cardiometabolic traits. Genetic (ρ_G) and environmental (ρ_E) covariance estimates, from the twin pair studies above, are illustrated graphically, as mean ± SEM for each covariance, with *P*-value for its significance.

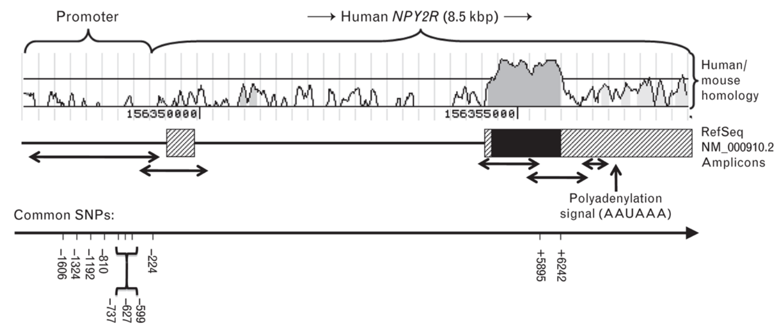

FIGURE 2.

NPY2R resequencing strategy and identified variants. Sequences conserved between mouse and human NPY2R were visualized with VISTA (http://genome.lbl.gov/vista/index.shtml). Locations of common (minor allele frequency   5%) single nucleotide polymorphisms (SNPs) are indicated. Positions are numbered upstream (–) or downstream (+) of the CAP (transcription initiation) site. Solid blocks, open reading frame (ORF); hatched blocks, UTRs. Bi-directional horizontal arrows, resequencing amplicons, with sequencing direction(s) indicated by arrowheads.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

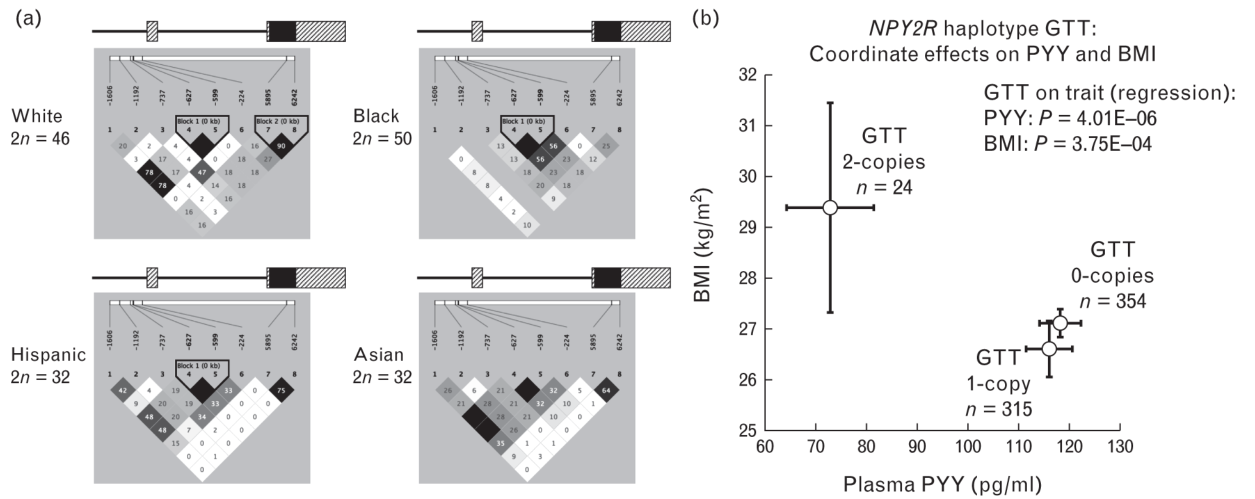

FIGURE 3.

Haplotype analyses at the *NPY2R* locus. (a) Linkage disequilibrium (LD) blocks across *NPY2R* in several biogeographic ancestry groups, derived by confidence intervals in Haploview. Numerical values shown in diamonds are $r^2 \times 100$. r^2 color scheme: $r^2 = 0$, white; $0 < r^2 < 1$, shades of grey; $r^2 = 1$, black. Common single nucleotide polymorphisms (SNPs): MAF 5%. Rectangles, exons; Diagonal shading, noncoding (UTR); Solid shading, coding (open reading frame). (b) Polymorphism at *NPY2R* influences human cardiometabolic traits: haplotype effects across the locus, 'tagged' by three SNPs (see Fig. 1 rs6851222 (Promoter G-1606A), rs6857715 (Promoter C-599T), and rs1047214 (Exon-2T/C Ile195Ile), chosen to span the *NPY2R* locus. Each SNP was in Hardy–Weinberg equilibrium (all $P > 0.05$). Both BMI and circulating peptide YY (PYY) were significantly influenced by the GTT haplotype, and the effects displayed evidence of joint determination (genetic pleiotropy).

NIH-PA Author Manuscript

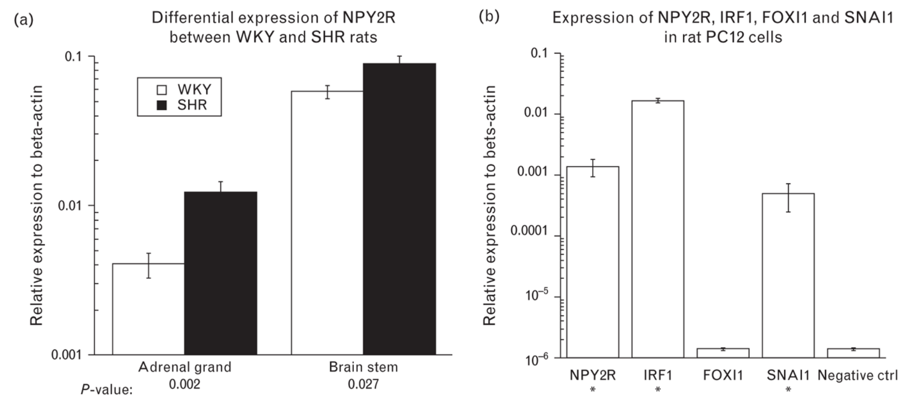

FIGURE 4.

Transcript (mRNA) expression for the *NPY2R* system, in tissues *in vivo* as well as in cultured neuroendocrine cells. Results were obtained by RT-PCR, using the $\Delta\Delta C_t$ method for normalization and condition comparisons. (a) Experimental (genetic) hypertension, differential expression of *NPY2R* in SHR and WKY neuroendocrine tissues (adrenal gland and brainstem). WKY, Wistar–Kyoto rat as a normotensive control; SHR, spontaneously hypertensive rat as a polygenic hypertension model. $N=9$ in each group. (b) Cultured neuroendocrine cells: presence of transcripts for *NPY2R* as well as transcription factors *IRF1, FOXI1,* and *SNAI1* in rat PC12 cells. The negative control (human elastin gene) was measured to define the threshold of expression. Genes with significantly higher expression than negative control are marked by an asterisk (*). $N=4$ samples in each group.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

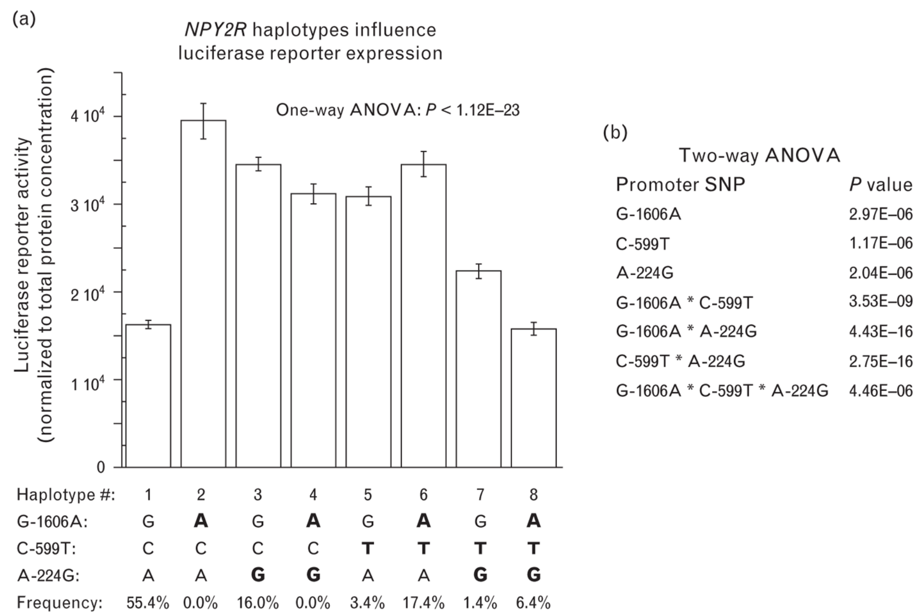

FIGURE 5.

NPY2R haplotypes influence luciferase reporter expression. (a) Eight haplotypes constructed by the combination of three single nucleotide polymorphisms (SNPs) have significantly different effects on expression of the reporter (one-way ANOVA: P=1.12E–23). The minor allele for each SNP is shown in bold. Frequency of each haplotype in our resequencing sample is shown at the bottom. Results for n=8 groups are shown. (b) Single SNPs and their binary and ternary interactions were examined using two-way ANOVA, and found to all have significant effects on reporter expression.

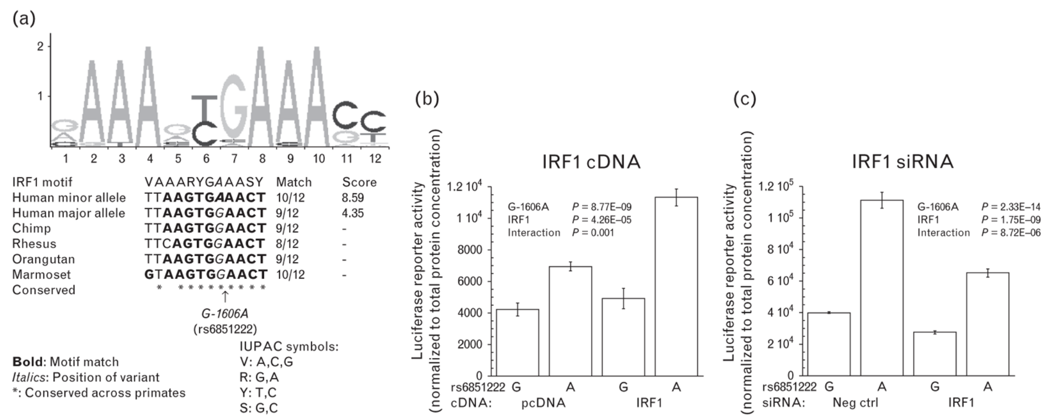

FIGURE 6.

NPY2R promoter variant G-1606A: role of IRF1. (a) Consensus motif match for IRF1 (interferon regulatory factor-1) at G-1606A across primate species. (b) Exogenous IRF1 cDNA enhanced reporter expression driven by NPY2R promoter, significantly more on the −1606A allele. (c) Exogenous IRF1 siRNA impaired the activator function of IRF1 on reporter expression driven by NPY2R promoter, significantly more on the −1606A allele. N =6 in each group.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

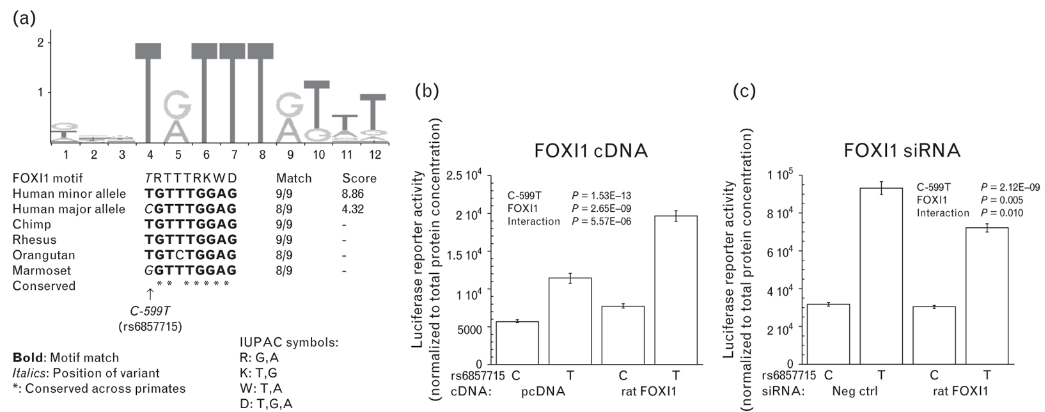

FIGURE 7.

NPY2R promoter variant C-599T: role of FOXI1. (a) Consensus motif match for FOXI1 (Forkhead Box I-1) at C-599T across primate species. (b) Exogenous FOXI1 cDNA enhanced reporter expression driven by NPY2R promoter, significantly more on the −599T allele. (c) Exogenous FOXI1 siRNA impaired the activator function of FOXI1 on reporter expression driven by NPY2R promoter, significantly more on the −599T allele. N =6 in each group.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

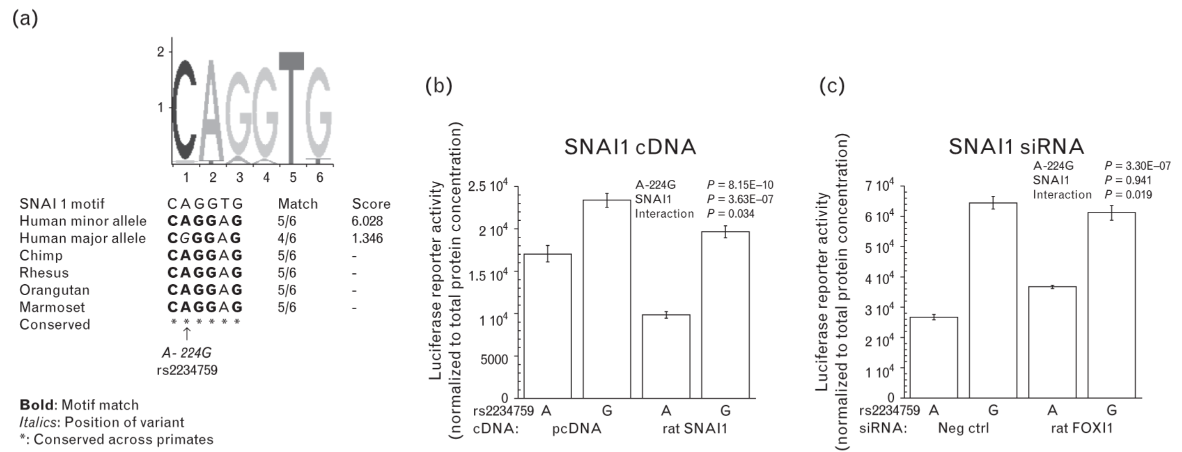

FIGURE 8.

NPY2R promoter variant A-224G: role of SNAI1. (a) Consensus motif match for SNAI1 (Snail homolog 1) at A-224G across primate species. (b) Exogenous SNAI1 cDNA inhibited reporter expression driven by NPY2R promoter, significantly more on the A-224 allele. (c) Exogenous SNAI1 siRNA impaired the repressor function of IRF1 on reporter expression driven by NPY2R promoter, significantly more on the A-224 allele. N =6 in each group.

NIH-PA Author Manuscript

TABLE 1

NPY2R polymorphism discovery in $n = 80$ individuals (i.e., $2n = 160$ chromosomes) from four biogeographic ancestries

					Minor allele frequency				
NPY2R SNPs	**Alleles**	**SNP position**	**RefSNP number**	**Amino acid change**	**White $n = 23$**	**Black $n = 25$**	**Hispanic $n = 16$**	**Asian $n = 16$**	**All $n = 80$**
1	A/G	−1637, promoter	rs57869523	none	–	–	–	–	0.018
2	G/A	−1606, promoter	rs6851222	none	0.25	0.16	0.25	0.47	0.270
3	T/C	−1449, promoter	rs10212938	none	–	–	–	–	0.029
4	–/T	−1324, promoter	rs36032070	none	0.25	0.22	0.25	0.47	0.290
5	G/A	−1192, promoter	rs33977152	none	0.11	0.00	0.12	0.19	0.090
6	–/GA	−810, promoter	rs35987718	none	0.45	0.12	0.38	0.25	0.287
7	–/AGAG	−807, promoter	rs34874489	none	–	–	–	–	0.017
8	A/T	−737, promoter	rs12507396	none	0.12	0.08	0.22	0.20	0.140
9	A/G	−627, promoter	rs6857530	none	0.29	0.65	0.41	0.47	0.470
10	C/T	−599, promoter	rs6857715	none	0.29	0.36	0.41	0.47	0.470
11	C/A	−314, promoter	NA	none	–	–	–	–	0.011
12	C/G	−265, promoter	NA	none	–	–	–	–	0.018
13	C/T	−257, promoter	rs73855386	none	–	–	–	–	0.018
14	A/G	−224, promoter	**rs2234759**	none	0.24	0.20	0.33	0.53	0.310
15	G/A	−220, promoter	NA	none	–	–	–	–	0.047
16	G/C	−186, promoter	NA	none	–	–	–	–	0.005
17	C/T	+85, exon-1 (5′-UTR)	NA	none	–	–	–	–	0.005
18	C/T	+324, exon-1 (5′-UTR)	rs72972775	none	–	–	–	–	0.029
19	T/C	+5469, exon-2	rs2342674	L-53-L	–	–	–	–	0.017
20	T/C	+5895, exon-2	*rs1047214*	I-195-I	0.48	0.12	0.34	0.07	0.250
21	T/C	+6242, exon-2	rs2880415	I-312-I	0.50	0.33	0.43	0.13	0.360

RefSNP, reference SNP; SNP, single nucleotide polymorphism. Positions and allele frequencies for each common (global frequency 5%) and rare (global frequency <5%) polymorphism is represented by population. Ethnicity-specific frequencies are given if overall/global MAF is >5%. Polymorphisms in the promoter region (upstream from the cap site) are numbered (–) and those downstream from the cap site are positive (+). A RefSNP number from NCBI is given if available in the public database. SNPs are represented as major (upper case) and minor (lower case) alleles. The high-frequency G-1606A, C-599T and A-224G promoter variants, which we focused on during molecular biology experiments are marked with **bold** RefSNP numbers. Italics, three variants chosen for inclusion in haplotypes to span the *NPY2R* locus for clinical marker-on-trait associations: rs6851222 (Promoter G-1606A), rs6857715 (Promoter C-599T), and rs1047214 (Exon-2 T/C Ile195Ile).