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Neuropeptide Y_1 Receptor NPY1R

Discovery of Naturally Occurring Human Genetic Variants Governing Gene Expression In Cella as Well as Pleiotropic Effects on Autonomic Activity and Blood Pressure In Vivo

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Objectives We asked whether naturally occurring genetic variation at the human *NPY1R* locus alters autonomic traits that might predispose individuals to cardiovascular disease.

Background Neuropeptide Y (NPY) interacts with the Y_1 receptor, NPY1R, to control adrenergic activity and blood pressure (BP).

Methods We searched for polymorphism at *NPY1R* by systematic resequencing in ethnically diverse people. There were 376 twins/siblings who were evaluated for heritable autonomic traits: baroreflex function and pressor response to environmental stress.

Results The common *NPY1R* variant A+1050G in the 3′-untranslated region (3′-UTR) predicted baroreceptor slope ($p = 0.014–0.047$) and BP change to cold stress ($p = 0.0091–0.016$), with minor allele homozygotes displaying blunted slope and exaggerated pressor response. In 936 individuals with the most extreme BPs in the population, not only 3′-UTR A+1050G ($p = 1.2 \times 10^{-4}$) but also promoter A-585T ($p = 0.001$) affected both systolic BP and diastolic BP, in interactive fashion ($p = 0.007$), with combined homozygotes showing the highest diastolic BP (>20 mm Hg). The 3′-UTR variant +1050G decreased expression of a transfected luciferase reporter/*NPY1R* 3′-UTR plasmid; promoter variant A-585 also decreased expression of an *NPY1R* promoter/luciferase reporter. Thus, alleles that increased BP in vivo (3′-UTR +1050G, promoter A-585) also decreased NPY1R expression in cella. Computational alignment showed that A+1050G disrupted a microRNA motif.

Conclusions Our results indicate that naturally occurring genetic variation at the *NPY1R* locus has implications for heritable autonomic control of the circulation, and ultimately, for systemic hypertension. The findings suggest novel pathophysiological links between the *NPY1R* locus, autonomic activity, and blood pressure, and suggest new strategies to approach the mechanism, diagnosis, and treatment of systemic hypertension. (J Am Coll Cardiol 2009; 54:944–54) © 2009 by the American College of Cardiology Foundation

Systemic hypertension is a common though complex trait that displays heritable alterations in autonomic control of the circulation (1,2). Neuropeptide Y (NPY) is a sympathetic cotransmitter along with catecholamines (3); NPY accounts for a unique spectrum of physiological actions in both the central and peripheral nervous systems, including cardiovascular homeostasis (4), hormone secretion (5), blood pressure (BP) (6), the stress response, emotion (7), and feeding behavior (8). The NPY receptor family has several members; in contrast to the Y_2, Y_4, Y_5, and Y_6 receptor isoforms, the Y_1 receptor (NPY1R) preferentially binds NPY. The gene encoding the *NPY1R* G-protein coupled receptor is located on chromosome 4q31.3-q32 (9).

NPY acts in the periphery, via the NPY1R receptor, to exert a vasoconstrictor/pressor effect (10); by contrast, NPY/NPY1R actions in the central nervous system may be

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antihypertensive (6). Rodents transgenic for NPY overexpression display blunted BP elevations after central nitric oxide synthase inhibition, a phenomenon that can be reversed centrally by the selective Y_1 receptor antagonist BIBP3226 (6). In addition, *NPY1R* knockout mice display profound changes in adrenergic activity, with increased catecholamine biosynthesis and secretion (11). However, the role of *NPY1R* variation in human BP regulation remains unexplored.

Considering the potential effects of *NPY1R* variation on human BP, we hypothesized that human genetic variation at *NPY1R* may result in quantitative or qualitative changes in NPY1R activity, in turn influencing NPY signaling so as to alter autonomic activity and perhaps predispose to cardiovascular disease. We therefore explored natural allelic variation at the *NPY1R* locus by resequencing the gene in 80 ethnically diverse subjects (2n = 160 chromosomes). We then explored the role of *NPY1R* genetic variation in human physiology, with a twin cohort extensively phenotyped for autonomic activity as well as disease, using a sample of individuals with the most extreme BPs in the population. Finally, we characterized the functional activity of 2 disease-associated variants in the 3′-untranslated region (UTR) and proximal promoter.

Abbreviations and Acronyms

BP = blood pressure
DBP = diastolic blood pressure
Del = deletion
h^2 = heritability
HR = heart rate
Ins = insertion
LD = linkage disequilibrium
NPY = neuropeptide Y
NPY1R = neuropeptide Y_1 receptor
SBP = systolic blood pressure
SNP = single nucleotide polymorphism
UTR = untranslated region

Methods

Subjects and clinical characterization. POLYMORPHISM DISCOVERY AND BIOGEOGRAPHIC ANCESTRY. Initially, 80 individuals (2n = 160 chromosomes) were studied by resequencing of *NPY1R* for systematic polymorphism discovery: 20 individuals were of European ancestry (white), 20 were of sub-Saharan African ancestry (black), 20 were of Asian ancestry, and 20 were of Hispanic ancestry. Ethnicity was established by self-identification by the participants as well as their parents and grandparents. None of the subjects had a history of renal failure. Definitions of subject characteristics are according to previous reports from our laboratory (12). Subjects were volunteers from southern California, and each subject gave informed and written consent; the protocol was approved by the University of California San Diego Institutional Review Board.

TWIN/SIBLING SAMPLE. We recruited a series of twin pairs and siblings (n = 376), taking advantage of a large population-based twin registry in southern California (13,14) and newspaper advertising (14). These twin individuals were all of European ancestry to permit allelic association studies within 1 ethnicity. There were 360 twin individuals from 180 white (European ancestry) twin pairs, including 125 monozygotic pairs and 55 dizygotic pairs. The twin/sibling subjects were 14 to 84 years old.

HYPERTENSION. We studied 936 (404 male, 532 female) white (European ancestry) subjects, recruited from a large primary care (Kaiser Permanente) population in San Diego, as previously described (15). In this primary care population, approximately 81% attended the clinic, and approximately 46% consented to participation in the study, with collection of blood for preparation of genomic deoxyribonucleic acid (DNA). From consenting participants, the subjects in this study were selected, based upon measurement of diastolic blood pressure (DBP), to represent the highest and lowest fifths of diastolic BP percentiles in that population. There were 426 subjects chosen for higher DBPs; 510 subjects were chosen for lower DBPs. The DBP criterion was chosen because of its heritability (13). The statistical power of association between biallelic DNA markers and human quantitative trait loci can be substantially augmented by sampling individuals from opposite (upper and lower) ends of the trait distribution (16–18), and analyses of the quantitative trait in extreme subjects (as opposed to dichotomization of the trait) further enhances power (19). This population sample afforded us >90% power to detect genotype association with a trait when the genotype contributes as little as 2.5% to the total variation in males (even at $p < 10^{-8}$); the power is even higher in females. To accomplish this, individuals with lower BP (systolic blood pressure [SBP]/DBP = 108 ± 0.7/56 ± 0.2 mm Hg) were selected from the bottom 4.8th percentiles of DBP; the higher BP group (SBP/DBP = 154 ± 0.8/99 ± 0.3 mm Hg) was selected from the top 4.9th percentiles of DBP. Both SBP and DBP differed significantly between the BP extreme groups ($p < 0.0001$). Although ambulatory BP monitoring would increase the accuracy of BP diagnosis and provide superior prediction of target organ damage (20), the very large epidemiologic scale of this primary care-based ascertainment precluded its use here. Forty-one percent of patients in the higher BP group were taking 1 or more antihypertensive medications (including 15% on diuretics and 19% on angiotensin-converting enzyme [ACE] inhibitors), and no patients in the lower BP group were on such treatment. In subsequent analyses, BP results were also adjusted for the effects of antihypertensive treatment by adding a fixed value (10/5 mm Hg) to each treated BP, as described (21). Although such adjustments are necessarily imperfect, their value is reinforced by restoration of familial (sibling/sibling) BP correlations (21); alternative adjustments based on specific medication categories (22) were not used.

Genomics. SYSTEMATIC POLYMORPHISM DISCOVERY BY RESEQUENCING. Genomic DNA was prepared from leukocytes in ethylenediaminetetraacetic acid-anticoagulated blood, using PureGene extraction columns (Gentra, Qiagen, Valencia, California) as described previously (23). Public draft human genome sequences were obtained from

the University of California, Santa Cruz Genome Bioinformatics Website and used as a scaffold for primer design. The base position numbers were from the National Center for Biotechnology Information *NPY1R* source clones NM_000909, NT_016354, and NP_000900. Promoter positions were numbered with upstream of (–) the *NPY1R* exon 1 start (cap) site. The following polymerase chain reaction primers were designed by primer 3 (19) to span approximately 1,000 bp of the proximal promoter and each of the 3 exons. Promoter 1, left primer 5′-ggcagtgccctgtatcttta-3′, right primer 5′-tgccactgtgcttttctttg-3′; promoter 2, left primer 5′-gccattattgtggcgaattt-3′, right primer 5′-tcgtttttcttcccctctca-3′; exon 1, left primer 5′-cagtatgttttcaccatttctgc-3′, right primer 5′-acagcaaggacccaaatcac-3′; exon 2, left primer 5′-cgagggtggagaccaaataa-3′, right primer 5′-catcgtggacatggctattg-3′; exon 3-1, left primer 5′-ccacgatgcacacagatgtt-3′, right primer 5′-tgacaatcagttgggagcaa-3′; exon 3-2, left primer 5′-agaagtggtttgaggtttct-3′, right primer 5′-gaaaaagcaaaaacaatattg-3′.

Target sequences were amplified by polymerase chain reaction from 20 ng genomic DNA in a final volume of 25 μl, which also contained 0.1 U of Taq DNA polymerase (Applied Biosystems, Foster City, California), 200 nM of each dNTP, 300 nM of each primer, 50 mM KCl, and 2 mM $MgCl_2$. Polymerase chain reaction was performed in an MJ PTC-225 thermal cycler, starting with 12 min of denaturation at 95°C, followed by 45 cycles at 95°C for 30 s, 63°C for 1 min (annealing), 72°C (extension) for 1 min, and then a final extension of 8 min at 72°C. Polymerase chain reaction products were treated with exonuclease I and shrimp alkaline phosphatase to remove primers and then dNTPs before cycle sequencing with BigDye terminators (Applied Biosystems). Sequence was determined on an ABI-3100 automated sequencer. Polymorphism and heterozygosity were detected using a CodonCode Aligner (CodonCode Corp., Dedham, Massachusetts).

SINGLE NUCLEOTIDE POLYMORPHISM (SNP) GENOTYPING. Genotypes were determined by 2 extension-based techniques, either the MALDI (matrix assisted laser desorption ionization) mass spectrometry method of Sequenom (San Diego, California), or the luminescent base incorporation method of Pyrosequencing (Biotage, Uppsala, Sweden), in which genotypes were verified by visual inspection, with exclusion of artifactual data from further analysis. Reproducibility of diploid genotypes was verified with blinded replicate samples, indicating 98.8% concordance.

Physiological/autonomic phenotyping in vivo. Noninvasive brachial arterial cuff BPs were obtained in seated subjects with an oscillometric device, as previously described (24). To probe autonomic control of the circulation in twin pairs, BP and heart rate were recorded continuously and noninvasively for 5 min in seated, resting subjects with a radial artery applanation device as well as thoracic electrocardiogram electrodes and dedicated sensor hardware (Colin Pilot, Colin Instruments, San Antonio, Texas) and software (ATLAS, WR Medical Electronics Co., Stillwater, Minnesota; and Autonomic Nervous System, Tonometric Data Analysis [ANS-TDA], Colin Instruments). Baroreceptor slope and heart rate variability were quantified as previously described (25). The BP and heart rate responses to environmental (cold) stress (1 hand immersed in ice water for 1 min) were obtained with the same hardware and software, as previously described (25).

***NPY1R* variants (promoter A-585T, and 3′-UTR A+1050G): Functional consequences in cella.** **3′-UTR.** The 1338 bp 3′-UTR was amplified with polymerase chain reaction primers incorporating XbaI restriction sites, facilitating ligation into the unique XbaI site in the luciferase reporter plasmid pGL3-Promoter (Promega, Madison, Wisconsin); in this plasmid, the XbaI site is just downstream (3′) of the firefly luciferase reporter open reading frame and upstream of the polyadenylation signal (pA from SV40). Transcription is driven by the SV40 early promoter, just upstream (5′) of the luciferase cassette. Correct orientation (5′ to 3′) of the insert was verified by sequencing.

PROMOTER. The 1101 bp promoter region was amplified with polymerase chain reaction primers incorporating NheI and HindIII restriction sites, facilitating ligation into the luciferase reporter plasmid pGL3-Basic (Promega). The insert was verified by sequencing.

MUTAGENESIS AND TRANSFECTION. Creation of the A+1050G or A-585T polymorphisms was achieved by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, California). After plasmid growth under ampicillin selection in *E. coli* and purification on columns (Qiagen), supercoiled plasmid DNA was transfected into PC12 chromaffin cells using established protocols and Superfect cationic lipid reagent (Qiagen), along with the transfection efficiency control plasmid p-cytomegalovirus-beta-Gal, encoding the beta-galactosidase reporter plasmid under control of the cytomegalovirus promoter (Promega). After transfection and cell growth over an 8- to 24-h time course, cells were lysed for sequential measurement of luciferase enzymatic activity and beta-galactosidase expression.

Statistical genetics. DESCRIPTIVE STATISTICS. Descriptive statistics (mean, SEM) were computed across all twins, using generalized estimating equations (PROC GENMOD) in Statistical Analysis System (SAS Institute, Cary, North Carolina). Population BP extreme analyses were performed by 2-way analysis of variance in the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois).

Heritability of phenotype expression in vivo. Heritability (h^2) is the fraction of phenotypic variance accounted for by genetic variance ($h^2 = V_G/V_P$). Estimates of h^2 were obtained using the variance component method implemented in the Sequential Oligogenic Linkage Analysis Routine (SOLAR) package (26). This method maximizes the likelihood of the estimate assuming a multivariate normal distribution of phenotypes in twin pairs (monozygotic vs. dizygotic) with a mean dependent on a particular

set of explanatory covariates. The null hypothesis (H_0) of no heritability ($h^2 = 0$) is tested by comparing the full model, which assumes genetic variation, and a reduced model, which assumes no genetic variation, using a likelihood ratio test. Covariates (sex and age) that were significant at $p < 0.05$ were retained in the heritability model. Before analyses in the Sequential Oligogenic Linkage Analysis Routine, exploratory descriptive statistics were computed for each trait, and if trait values displayed excessive kurtosis, they were $\log_{10}$-transformed to achieve kurtosis less than 0.8.

Haplotypes and linkage disequilibrium (LD). After resequencing, we constructed LD maps by using 4 common SNPs (minor allele frequencies >10%). Blocks of LD were displayed using Haploview (27), using the 4-gamete rule, and displaying pairwise results as r^2. Haplotypes constructed by A+1050G (3′-UTR) and A-585T (promoter) were inferred by the HAP algorithm (28).

Marker-on-trait association. During associations at A+1050G and A-585T (or their haplotypes), subjects were categorized according to either diploid genotype at a biallelic SNP locus, or carrier status (2, 1, or 0 copies) for a particular haplotype. When more than 1 SNP within an LD block was associated with a trait, the software SNPSpD (29) was used to yield new experiment-wide thresholds to maintain the type I error rate at ≤5%.

SNP-by-SNP interaction. We tested the interaction between A+1050G and A-585T by 2-way analysis of variance in SPSS. Diastolic BP value was taken as the dependent variable; both of the SNPs were categorized as fixed factors. Covariates were age and sex.

Bioinformatics. Multiple sequence alignments across species were conducted in Clustal-W http://www.ebi.ac.uk/Tools/clustalw2/index.html. 3′-UTR microRNA motifs were predicted at http://www.microrna.org/microrna/getGeneForm.do or at RegRNA http://bidlab.life.nctu.edu.tw/RegRNA2/website/. Ribonucleic acid (RNA) hybrid structures and predicted minimum folding energies were analyzed at http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html. RNA folding algorithms (http://www.genebee.msu.su/service/rna2_reduced.html) predicted folding free energy difference between wild-type and variant, as well as messenger ribonucleic acid (mRNA) stem/loop structures.

Results

Systematic human polymorphism discovery at *NPY1R*. To identify genetic variants in *NPY1R*, we resequenced both exons and adjacent regions, ~1 kbp of 5′ promoter region, and the UTRs in DNA from 80 human subjects (2n = 160 chromosomes) from 4 biogeographic ancestry groups: Asian, Hispanic, black, and white. Figure 1 and on-line Table 1 illustrate the strategy. Altogether, 12 biallelic SNPs and 1 (4-bp) insertion/deletion were identified. Among the 12 SNPs, 7 were located within the ~1 kbp proximal promoter, 1 in the 5′-UTR, 1 in the coding region, and 3 in the UTRs. Four variants were common (minor allele frequency >5%): 2 SNPs and the insertion/deletion (Ins/Del) in the promoter, and 2 in the 3′-UTR.

SNP genotype frequencies differed substantially by ethnicity (Online Table 1): Promoter T-674G occurred only in the black and Hispanic groups. The 3′-UTR T+601C was an unusual variant in Hispanics, but it was substantially more common in the other 3 groups. Although A-585T,

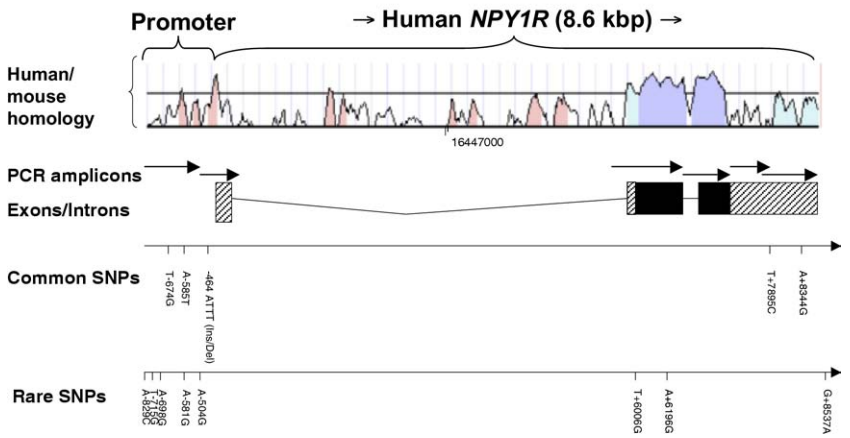

Figure 1 ***NPY1R* Resequencing Strategy and Identified Variants**

Sequences conserved between mouse and human neuropeptide Y_1 receptors (*NPY1R*) were visualized with Windows VISTA (Microsoft, Redmond, Washington). Locations of common (minor allele frequency >5%) and rare single nucleotide polymorphisms (SNPs) are indicated. Positions are numbered upstream or downstream of the transcriptional initiation (exon 1) start site. **Solid blocks** = open reading frame; **hatched blocks** = untranslated regions (UTRs). PCR = polymerase chain reaction.

Table 1 Heritability (h^2) and Effects of *NPY1R* 3′-UTR Variant A+1050G on Physiological Traits in Twin Siblings Pairs

Phenotype	Heritability (h^2) (360 Twins)		*NPY1R* 3′-UTR A+1050G Diploid Genotype (376 Twins and Sibs)			
	% ± SEM	p Value	A/A or A/G (n = 357)	G/G (n = 19)	Chi-Square	p Value
Baroreceptor coupling (frequency domain), ms/mm Hg						
At 0.15–0.40 Hz (high frequency)	41.2 ± 0.08	**<0.001**	15.7 ± 2.0	10.2 ± 1.8	5.90	**0.015**
At 0.05–0.15 Hz (low frequency)	52.6 ± 0.06	**<0.001**	15.1 ± 1.2	11.5 ± 1.5	3.97	**0.047**
Baroreceptor slope (time domain), ms/mm Hg						
Upward deflections	25.8 ± 0.10	**0.009**	15.0 ± 0.6	10.8 ± 1.4	5.99	**0.014**
Downward deflections	38.6 ± 0.07	**<0.001**	12.5 ± 0.6	9.7 ± 1.2	4.33	**0.037**
Cold stress response, mm Hg						
Initial SBP	40.7 ± 0.07	**<0.001**	118.9 ± 1.1	119.8 ± 2.9	0.11	0.739
Initial DBP	28.7 ± 0.09	**0.001**	64.6 ± 0.8	67.6 ± 2.0	2.11	0.146
Final SBP	28.3 ± 0.08	**<0.001**	131.1 ± 1.5	139.2 ± 4.0	3.29	0.069
Final DBP	30.1 ± 0.08	**<0.001**	75.5 ± 1.0	82.5 ± 2.1	6.72	**0.0095**
Change in SBP	19.8 ± 0.10	**0.02**	12.9 ± 1.4	20.2 ± 2.2	6.80	**0.0091**
Change in DBP	26.7 ± 0.09	**0.001**	10.9 ± 0.9	15.4 ± 1.6	5.77	**0.016**

Values of h^2 are mean % ± SEM, where h^2 is the % of phenotypic variation (V_P) explained by additive genetic factors (V_G), in 360 twins. Values are age- and sex-adjusted in Sequential Oligogenic Linkage Analysis Routine (SOLAR), and expressed as %. The p value is the significance of the heritability value. Single nucleotide polymorphism (SNP) statistics for twin and sibling study population were derived from generalized estimating equations (PROC GENMOD) in SAS, to account for correlations within families. **Bold** values indicate $p < 0.05$.

DBP = diastolic blood pressure; *NPYIR* = neuropeptide Y_1 receptor; SBP = systolic blood pressure.

[ATTT/-] −464 Ins/Del, and A+8344G (within the 3′-UTR; A+1050G) were common in all 4 ethnic groups, their frequencies differed among the groups.

Linkage disequilibrium (LD) across the *NPY1R* locus. Pairwise LD among 4 common SNPs and the Ins/Del across *NPY1R* was qualified as parameter r2, scaled from 0 to 1. Online Figure 1 shows the LD and haplotype block structure in 4 different ethnic groups. In the promoter, [ATTT/-] −464 Ins/Del and A-585T were in complete LD in whites and Asians, and strong LD in blacks and Hispanics. In Asians, the 4 common polymorphisms were located within a single LD block (by the 4-gamete rule in Haploview); additional blocks are apparent in the other groups.

Autonomic trait heritability (h^2) in twins. Heritability (Table 1) was significant for several autonomic traits, such as the hemodynamic response to environmental (cold) stress, including initial SBP ($h^2 = 40.7 \pm 7.3\%$, $p < 0.0001$), initial DBP ($h^2 = 28.7 \pm 8.6\%$, $p = 0.001$), final SBP ($h^2 = 28.3 \pm 7.9\%$, $p = 0.0004$), final DBP ($h^2 = 30.1 \pm 7.8\%$, $p = 0.0001$), DBP change ($h^2 = 26.7 \pm 8.5\%$, $p = 0.0014$), and SBP change ($h^2 = 19.8 \pm 9.7\%$, $p = 0.023$). Heritability was also significant for baroreceptor coupling in the low frequency domain (0.05 to 0.15 Hz; $h^2 = 52.6 \pm 5.9\%$, $p < 0.0001$), high frequency domain (0.15 to 0.4 Hz; $h^2 = 41.2 \pm 7.5\%$, $p < 0.0001$), and for baroreceptor slope, both upward deflections ($h^2 = 25.8 \pm 10.5\%$, $p = 0.009$), and downward deflections ($h^2 = 38.6 \pm 7.2\%$, $p < 0.0001$).

***NPY1R* variant association with autonomic traits in twins.** We began by testing whether the *NPY1R* genetic variation affected physiological traits in twins. Since the number of LD blocks was limited (Online Fig. 1), we chose 2 common SNPs to "tag" the locus: promoter/A-585T and 3′-UTR/A+1050G. The 3′-UTR variant A+1050G predicted several physiological traits whose alterations are involved in the pathogenesis of hypertension (1).

BARORECEPTOR MECHANISM. The baroreceptor system exerts second-to-second feedback control over blood pressure homeostasis, and baroreceptor defects may precede hypertension, as early "intermediate phenotypes" (1). The 3′-UTR variant predicted baroreceptor slope, during both upward deflections ($p = 0.014$) and downward deflections ($p = 0.037$). 3′-UTR A+1050G also predicted beat-to-beat baroreflex coupling, in both the high frequency (0.15 to 0.40 Hz, $p = 0.015$) and low frequency (0.05 to 0.15 Hz, $p = 0.047$) domains (Table 1).

PRESSOR RESPONSE TO ENVIRONMENTAL (COLD) STRESS. Longitudinal studies of the BP response to cold stress (cold pressor test) indicate that change in BP may predict future development of hypertension (30). Significant prediction by A+1050G was observed for not only final (post-cold) DBP ($p = 0.0095$), but also for SBP ($p = 0.0091$) and DBP ($p = 0.016$) changes during cold stress.

In Figure 2, the relationship between baroreceptor slope (upward deflections) and SBP change to cold stress is portrayed in 2 dimensions. The 3′-UTR A+1050G minor allele homozygotes (G/G diploid genotype) displayed decreased baroreceptor slope and increased SBP change. Thus, the G allele seems to act recessively on these traits.

Neither the promoter/A-585T nor the 3′-UTR/A+1050G variants were associated with body mass index in twins (for promoter/A-585T, $p = 0.81$; for 3′-UTR/A+1050G, $p = 0.74$).

***NPY1R* polymorphisms and hypertension.** The 3′-UTR A+1050G and promoter A-585T were scored in 936 individuals selected from the extreme upper/lower fifth percentiles of BP in a large primary care population (Online Table 1). Each SNP showed significant effects on both DBP (Fig. 3) (A-585T, $p = 1.2 \times 10^{-4}$; A+1050G, $p = 0.001$) and SBP (A-585T, $p = 0.018$; A+1050G, $p = 0.012$).

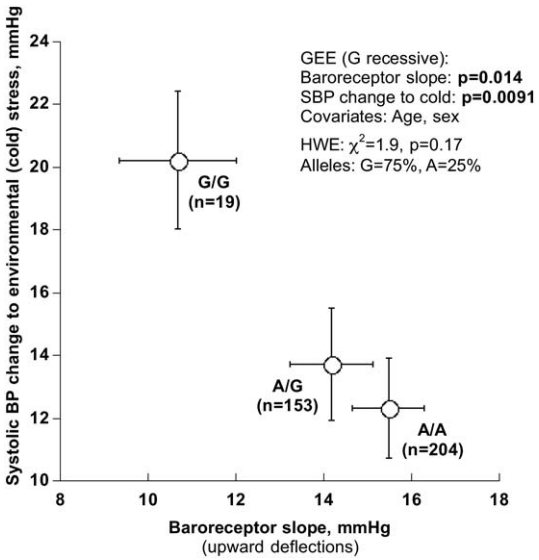

Figure 2 ***NPY1R* 3′-UTR Variant A+1050G: Pleiotropic Effects on Coupling Between Baroreceptors and Stress Response**

BP = blood pressure; GEE = generalized estimating equation; other abbreviations as in Figure 1.

In addition, there was an SNP-by-SNP interaction on DBP (SNP-by-SNP p = 0.007): A+1050G effects on BP depended upon the background of A-585T genotype, with the very highest DBP values found in subjects with combined -585 A/A and +1050 G/G homozygosity. Significance did not change after BP adjustment for treatment.

BP was also analyzed as a dichotomous trait (high vs. low), using both 2-way analysis of variance and regression. On analysis of variance, BP status was influenced by promoter A-585T ($p = 3.92 \times 10^{-4}$), as well as 3′-UTR A+1050G (p = 0.005), and the interaction of the 2 (p = 0.032). On regression, BP status was influenced by promoter A-585T (p = 0.001) as well as 3′-UTR A+1050G (p = 0.030).

On 2-SNP haplotype analyses across *NPY1R*, Hap-4 (promoter A-585 → 3′-UTR +1050G) was strongly associated with both SBP and DBP (Fig. 4): subjects with 2 copies of Hap-4 displayed increases in both SBP and DBP (SBP, p = 0.007; DBP, $p = 2.34 \times 10^{-5}$). When BP was analyzed as a dichotomous trait (Fisher's exact test), Hap-4 copy number affected the trait ($p = 2.3 \times 10^{-4}$).

Although the hypertensive subjects displayed higher body mass index than the normotensive group (30.6 ± 0.3 vs. 24.6 ± 0.2 kg/m², p < 0.001) (Online Table 1), neither of the 2 NPY1R variants associated with body mass index in this sample (promoter/A-585T, p = 0.096; 3′-UTR/A+1050G, p = 0.918).

Statistics: alpha threshold. We re-evaluated the alpha threshold required to avoid false-positive conclusions in the setting of 2 genetic markers (3′-UTR A+1050G and promoter A-585T) by using SNPSpD (SNP SPectral Decomposition) (29), which takes into account the correlations among linked markers. The effective number of markers was computed at 1.76, and thus the appropriate alpha threshold fell to p = 0.028. The polymorphism effects on twin traits and population BP exceeded even this more stringent threshold.

***NPY1R* 3′-UTR A+1050G and promoter A-585T: functional consequences in cella and interspecies sequence homology.** 3′-UTR A+1050G. Wild-type (A+1050) versus variant (+1050G) 1338 bp *NPY1R* 3′-UTRs were ligated into reporter plasmid pGL3-Promoter, downstream of the luciferase reporter gene. After transfection into PC12 cells, cellular luciferase activity was measured. The 2 3′-UTR plasmids had significantly different luciferase activities in chromaffin cells, with wild-type A > variant G at several time points after transfection (p < 0.001) (Fig. 5); the A>G difference was also seen in embryonic kidney cells (Online Fig. 2) (p < 0.001). In HeLa fibroblasts, the activities of both promoters were diminished, though the A>G difference persisted.

Promoter A-585T. Promoter A-585T was in complete linkage disequilibrium (r2 = 1) with the −464[ATTT] Ins/Del in the white population: the A-585 allele appeared consistently with −464ATTT Ins; the −585T allele appeared with −464ATTT Del. Thus, wild-type (A-585 → −464ATTT Ins) versus variant (−585T → −464ATTT Del) 1101 bp *NPY1R* promoter segments were ligated into reporter plasmid pGL3-Basic, just upstream of the luciferase reporter gene. We also used site-directed mutagenesis to create all 4 possible promoter haplotypes. After transfec-

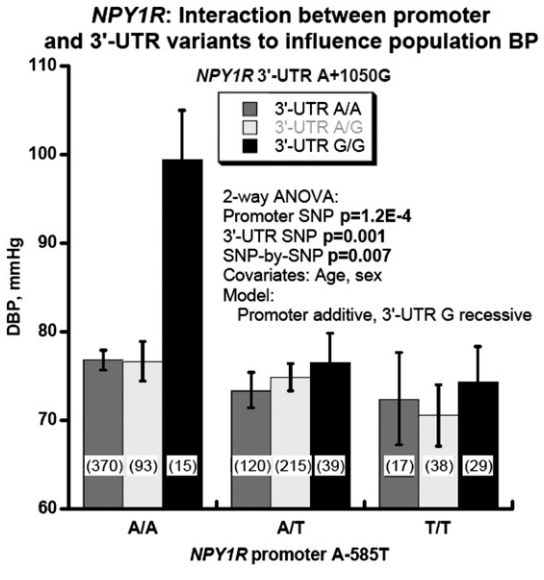

Figure 3 ***NPY1R* Common Variants in the 3′-UTR (A+1050G) and Proximal Promoter (A-585T) Influence BP Both Independently and Interactively**

ANOVA = analysis of variance; BP = blood pressure; DBP = diastolic blood pressure; other abbreviations as in Figure 1.

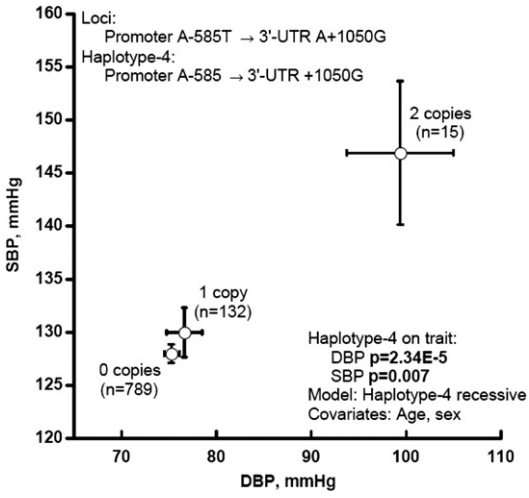

Figure 4 ***NPY1R* Haplotype 4 (A-585 → +1050G) Is Associated With Both DBP and SBP in a European Ancestry Population**

SBP = systolic blood pressure; other abbreviations as in Figure 1.

tion into PC12 cells for 24 h, luciferase activity was measured.

The promoter haplotypes displayed significantly different luciferase activities ($p < 0.001$) (Fig. 6). Between the 2 naturally occurring haplotypes, the variant (−585T → −464ATTT Del) was ~25% more active ($p < 0.001$) than wild-type (A-585 → −464ATTT. Availability of all 4 haploytpes (both natural and artificial) allowed us to explore the contingent actions of each variant (Fig. 6): on a background of the −464 Del allele, −585T was more active

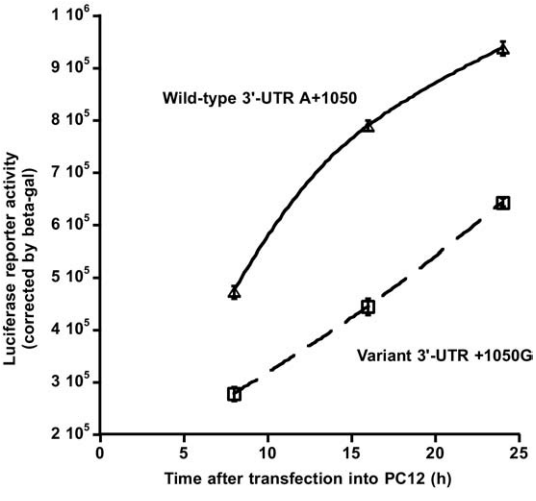

Figure 5 ***NPY1R* 3′-UTR Polymorphism A+1050G Influences Gene Expression In Cella**

Results of luciferase reporter/3′-UTR variant transfections into PC12 cells are shown. Abbreviations as in Figure 1.

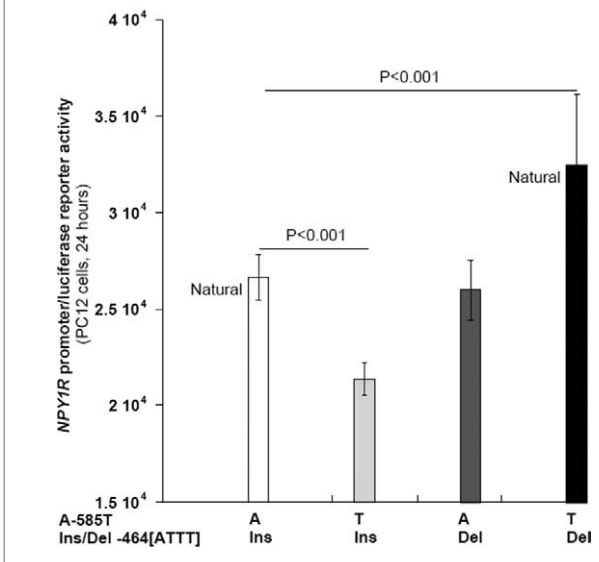

Figure 6 **Effect of *NPY1R* Common Promoter Variants (A-585T and Ins/Del) on Gene Expression In Cella**

Natural: Haplotype combination found in human *NPY1R* promoter deoxyribonucleic acid. Del = deletion; Ins = insertion; other abbreviations as in Figure 1.

than A-585; by contrast, on a background of the −464 Ins allele, A-585 was more active than −585T. Thus, both polymorphisms (A-585T and Ins-464Del) influenced transcriptional activity.

ALIGNMENTS. Both the promoter A-585T and 3′-UTR A+1050G polymorphisms lie in regions of generally conserved sequence across primate species (Online Figs. 3A and 3B). Based on the chimpanzee sequence, the likely human ancestral allele at A+1050G is A (the most frequent allele); at A-585T the likely ancestral allele is A (also the most frequent allele). Although the promoter region spanning A-585T was well conserved, no clear cut match was found for any known transcriptional control motif spanning A-585T.

***NPY1R* 3′-UTR A+1050G: RNA motifs and microRNA binding site prediction.** Sequence alignment suggested that A+1050G is located in a region where hsa-miR-511 interacts (Fig. 7). The RNA hybrid structure of hsa-miR-511 differed between A+1050 and +1050G, as did the predicted minimum folding energy of hsa-miR-511 hybridization. The lower minimum folding energy of the +1050G variant (at −24.4 kcal/mol) would predict better binding of hsa-miR-511 to the variant mRNA, and hence more efficient degradation of the variant mRNA, a finding consistent with decreased reporter expression by the variant luciferase/3′-UTR plasmid (Fig. 5).

Discussion

Overview. In this study, we probed whether common genetic variation at the NPY vasoconstrictive receptor (*NPY1R*) locus might influence autonomic function and

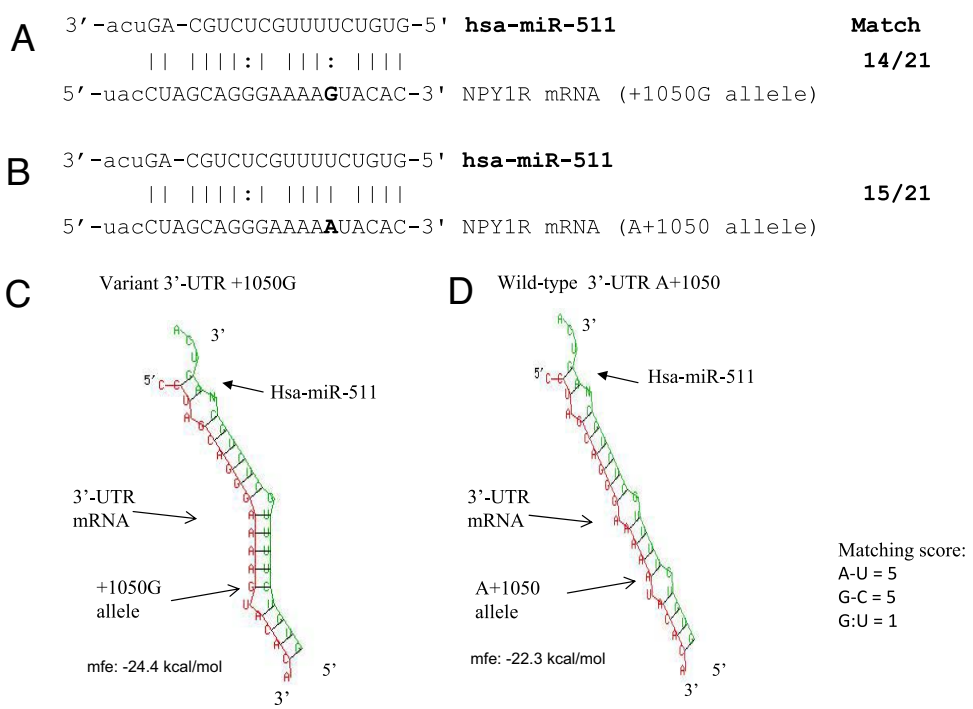

Figure 7 ***NPY1R* 3′-UTR Variant A+1050G Occurs in a Micro-RNA Recognition Motif**

(A, B) Complementarity between hsa-miR-511 and the *NPY1R* 3′-UTR site (1034–1055 bp downstream from the *NPY1R* stop codon), variant **(A)** versus wild-type **(B)**. The A+1050G SNP is given in bold type. Upper case: maximal scoring alignment region. Lowercase: not part of maximal scoring alignment region. **(C, D)** Hybridization of miR-155 to the *NPY1R* mRNA harboring the A **(D)** or G **(C)** alleles at A+1050G. **Arrows** highlight the polymorphic site. Calculated mfe= minimum free energy. Abbreviations as in Figure 1.

disease. We began by undertaking systematic polymorphisms discovery at *NPY1R*, revealing several novel variants in likely functional domains, especially noncoding regions. In a study of twin pairs characterized for heritable "intermediate" phenotypes that may be precursors to hypertension, a common variant in the 3′-UTR (A+1050G) was found to influence such traits, including baroreceptor function and the BP response to environmental stress. In subjects with the most extreme BP values in a large primary care population, 3′-UTR A+1050G and promoter A-585T interacted to determine BP. Finally, both the 3′-UTR and promoter variants were functional in luciferase reporter assays. The results indicate that common functional *NPY1R* polymorphisms affect *NPY1R* expression through both transcriptional and posttranscriptional mechanisms, eventuating in alterations of autonomic function, and ultimately, in systemic hypertension.

***NPY1R* distribution and functional roles.** NPY exhibits a range of important physiological activities, including effects on psychomotor activity, central regulation of endocrine secretion, and potent vasoactive effects on the cardiovascular system (31). *NPY1R* is the widely expressed major subtype of NPY receptor, where it mediates vasoconstriction in response to peripheral NPY (32). In the central nervous system, studies of an NPY transgenic rat by Michalkiewicz et al. (6) concluded that the central NPY/NPY1R system provides an endogenous mechanism to reduce pressor responses; hence peripheral and central NPY/NPY1R responses lead to diametrically opposite effects on BP.

Cavadas et al. (11) studied the effects of *NPY1R* knockout (targeted gene ablation): NPY caused secretion of catecholamines from primary cultures of mouse adrenal chromaffin cells, and this release was abolished in cultures from *NPY1R* knockout mice, suggesting an important role of the NPY/NPY1R signaling pathway toward catecholamine secretion. In *NPY1R* knockout mice they also found increases in adrenal catecholamine content, constitutive catecholamine release, and plasma catecholamine concentrations. In Y_1receptor-expressing cells, NPY decreased the activity of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis (11); such effects could be prevented by the selective Y_1 receptor antagonist BIBP3226, suggesting a far-reaching influence of the NPY/NPY1R system upon catecholamine metabolism.

Cardiovascular consequences of *NPY1R* polymorphism: autonomic and risk traits. *NPY1R* 3′-UTR A+1050G predicted several heritable (Table 1) autonomic traits that may be "intermediate phenotypes," or precursors to hypertension (1). In particular, 3′-UTR G/G homozygotes displayed not only diminished baroreflex function (by 3 indi-

ces) (Table 1), but also increased pressor responses to stress. Diminished baroreceptor function results in an inability to defend BP against such insults as environmental (e.g., cold) stress, and repeated pressor responses may eventuate in sustained or fixed BP elevation (30). Thus both baroreceptor dysfunction and increased BP pressor responses may be risk indicators for future development of hypertension (1,30). Of note for the Y_1 receptor, *NPY1R* knockout mice display hyperalgesia to thermal, cutaneous, and visceral chemical stimuli (31), although their pressor responses to cold stimulation are reportedly unchanged (33).

Finally, our genetic results suggest a role for a specific variant, *NPY1R* A+1050G, in triggering a pleiotropic series of effects on cardiovascular risk (Figs. 2 to 4); furthermore, phenotypic clustering as a function of genotype for both baroreceptor function and pressor responses indicates that G/G homozygotes cluster substantially away from other individuals (Fig. 2), suggesting that the G (minor) allele may act recessively in this regard. The substantial frequency of the G (minor) allele (at 25% to 50%) (Online Table 1) allowed us to detect the G/G effect in sufficiently large population samples (376 twins and siblings).

Hypertension: genetic interaction. In subjects with the most extreme BPs in the population (Fig. 3), promoter A-585T and 3′-UTR A+1050G each had substantial effects upon both DBP and SBP. In addition, we found that these 2 variants interacted significantly ($p = 0.007$) to determine DBP. For example, the most dramatic elevation of DBP occurred in subjects who were homozygotes for both the promoter variant major allele (A/A at A-585T) and the 3′-UTR variant minor allele (G/G at A+1050G). The importance of particular SNP-by-SNP combinations was confirmed in haplotype analyses (Fig. 4), in which we found that Hap-4 (promoter A-585 → 3′-UTR +1050G) had a copy number-dependent effect on both SBP ($p = 0.001$) and DBP ($p = 0.001$), with the most extreme effects (>15/>20 mm Hg SBP/DBP) in individuals who bore 2 copies of A→G.

Genetic variation: role of alterations in gene expression. In both the promoter and the 3′-UTR, hypertension-associated *NPY1R* alleles decreased gene expression in cella, though by predictably different mechanisms.

3′-UTR. Endogenous microRNAs mediate gene silencing by binding to specific motifs within the 3′-UTRs of target mRNAs (34). In the human *NPY1R* transcript, bioinformatic analyses indicated that variant A+1050G is located in a motif recognized by hsa-miR-511 (Fig. 7). The RNA hybrid structures and minimum folding energies of the complexes between hsa-miR-511 and the 3′-UTRs suggest that hypertension-associated variant +1050G participates in a more stable complex than wild-type A+1050. Such stable binding between hsa-miR-511 and 3′-UTR +1050G may facilitate the RNA-induced silencing complex, thereby reducing *NPY1R* expression.

There is emerging precedent for functional variation in 3′-UTRs that may contribute to blood pressure regulation. A functional variant in the 3′-UTR of the angiotensin II receptor *AGTR1* disrupts a microRNA binding motif (34). We recently characterized functional variants that contribute to hypertension risk in the 3′-UTRs of chromogranin A (a protein catalytic in formation of catecholamine storage vesicles) (30) and GTP cyclohydrolase (rate-limiting in formation of pterin cofactors) (35).

PROMOTER. In the promoter, common variants A-585T and Ins/Del/-464 each demonstrated functional activity in luciferase reporter constructs (Fig. 6). Hypertension-associated promoter allele −585T decreased promoter expression, whether studied in isolation (Fig. 3) or in the context of its naturally occurring haplotype (Fig. 4). Although the local sequence region around A-585T is conserved across species (Online Fig. 3B), the precise motif in cis, and trans-acting factors, perturbed by A-585T are not yet understood.

Advantages and limitations of this study. TWIN PHENOTYPING. We used the classical twin design in the search for trait-associated polymorphisms (36). Multiple autonomic phenotypes were measured in the twins, which permitted estimation of trait heritability as well as definition of effects of particular genetic variants at *NPY1R* on such "intermediate phenotypes" (Table 1); dual documentation of heritability and association lends internal consistency to the approach.

COUPLING SYSTEMATIC POLYMORPHISM DISCOVERY WITH IN CELLA AND HAPLOTYPE APPROACHES. Rather than using HapMap "tagging" SNPs at *NPY1R*, we systematically scanned the locus for both common and unusual variants in functional gene regions (exons, UTRs, and promoter). This approach allowed us to associate novel variants in potentially active regions with physiological traits in vivo, and then pursue the role of such variants in reporter assays in cella (Figs. 5 and 6, Online Fig. 2). Haplotyping across the locus then allowed us to confirm that particular combinations of promoter and 3′-UTR alleles (especially promoter A-585 and 3′-UTR +1050G) achieved especially pronounced effects on BP in the population (Fig. 4).

CAVEATS. First, although we conducted systematic polymorphism discovery in 4 diverse ethnic groups, our studies on autonomic physiology and disease were conducted only in subjects of European ancestry; whether the observed marker-on-trait associations occur in other populations await testing.

False positive (type I) statistical errors may occur during genetic associations, since the effects of multiple genetic variants may be evaluated on traits. First of all, we used haplotype analysis, and achieved a genetic effect on population BP (Fig. 4) without multiple testing. Second, we applied SNPSpD (29) to compute the effective number of SNPs tested and a new target alpha threshold; the modified

alpha = 0.028 was exceeded in our association tests. The effects of *NPY1R* variants on BP were confirmed in 2 independent samples (twin pairs and population BP extremes). Ultimately, our mechanistic studies in cella (Figs. 5 and 6, Online Fig. 2) lend plausibility to our results.

Finally, we found that *NPY1R* variants that decreased gene expression in cella predicted increased BP in vivo. Since the NPY system seems to exert opposite effects on BP when administered centrally versus peripherally (6), we have proposed a model in which the central effects of *NPY1R* polymorphism predominate on trait determination (Fig. 8). However, although the central effects of the NPY/NYP1R system can be explored in experimental animals (6), we cannot directly test the central effects of NPY/NPY1R activation in humans.

Conclusions and Perspectives

Common polymorphism occurs across the *NPY1R* locus, including variants in such active domains as the proximal promoter and 3′-UTR (Fig. 1). Such genetic variation, especially in the 3′-UTR, predicts interindividual variability in early, pathogenic autonomic traits that are precursors to development of hypertension, such as baroreceptor dysfunction and exaggerated pressor responses to stress (Fig. 2). *NPY1R* variants in both the promoter and 3′-UTR interact synergistically to influence BP in the population. Promoter and 3′-UTR variants were each functional during in cella luciferase reporter assays; the 3′-UTR variant disrupted a microRNA recognition motif. In each case (promoter and 3′-UTR), the variant that decreases *NPY1R* expression in cella also increases BP in vivo. Since central and peripheral NPY seem to exert directionally opposite effects on BP (6), we present a hypothetical mechanistic scheme (Fig. 8) that links these events in the most parsimonious longitudinal fashion. These results indicate novel pathophysiological links between *NPY1R* and blood pressure, and may provide new strategies for understanding the mechanism, diagnosis, and treatment of systemic hypertension.

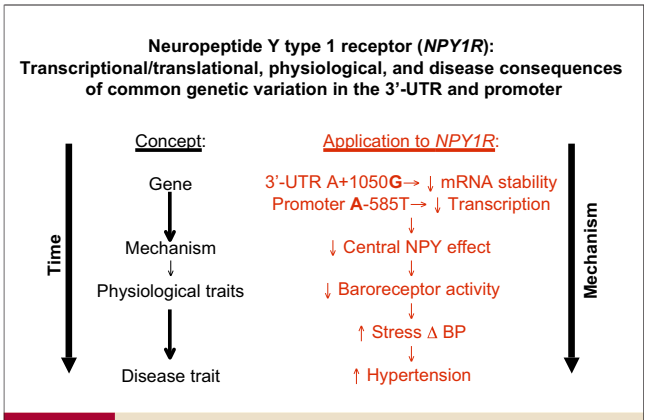

Figure 8 Neuropeptide Y Type 1 Receptor

Transcriptional/translational, physiological, and disease consequences of common genetic variation in the 3′-UTR and promoter. Abbreviations as in Figure 1.

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Key Words: hypertension ■ neuropeptide ■ genetics.

APPENDIX

For supplementary Tables 1 and 2 and supplementary Figures 1 to 4, please see the online version of this article.