Published in final edited form as:
J Am Coll Cardiol. 2009 September 1; 54(10): 944–954. doi:10.1016/j.jacc.2009.05.035.

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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Abstract

Background: Neuropeptide Y (NPY) interacts with the Y_1 receptor, NPY1R, to control adrenergic activity and BP. We asked whether naturally occurring genetic variation at the human *NPY1R* locus alters autonomic traits that might predispose individuals to cardiovascular disease.

Methods and Results: We identified polymorphisms in *NPY1R* by resequencing the gene in ethnically diverse people. 376 twins/siblings were evaluated for heritable autonomic traits: baroreflex function and pressor response to stress. Common *NPY1R* variant A+1050G in the 3′-untranslated region (3′-UTR) predicted baroreceptor (p=0.014-0.047) and BP change to environmental (cold) stress (p=0.0091-0.016), with minor allele homozygotes displaying blunted baroreceptor function and exaggerated pressor responses. In 936 individuals with the most extreme BPs in the population, not only 3′-UTR A+1050G (p=1.2E-4) but also promoter A-585T (p=0.001) affected both SBP and DBP, in interactive fashion (p=0.007), with combined homozygotes showing the highest DBP (>20 mmHg). 3′-UTR variant +1050G decreased reporter expression by a transfected luciferase reporter/*NPY1R* 3′-UTR expression plasmid, while promoter variant A-585 also decreased expression by an *NPY1R* promoter/luciferase reporter. Thus, the 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

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Conflicts of interest: None to disclose.

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.

Keywords

hypertension; neuropeptide; genetics

INTRODUCTION

Systemic hypertension is a common through complex trait that displays heritable alterations in autonomic control of the circulation(1;2). Neuropeptide Y (NPY) is a sympathetic co-transmitter 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(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 CNS may be *anti*-hypertensive(6). Rodents transgenic for NPY *over*-expression display *blunted* BP elevations after central NOS 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 two disease-associated variants in the 3′-UTR and proximal promoter.

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) of four biogeographic ancestry groups: Asian, Hispanic, Black and white. Figure 1 and on-line Table 1 illustrate the strategy. Altogether 12 biallelic SNPs and one (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 untranslated regions (UTRs). Four variants were common (minor allele frequency >5%): 2 SNPs and the insertion/deletion in the promoter, and 2 in the 3′-UTR.

SNP genotype frequencies differed substantially by ethnicity (on-line Table 1): Promoter T-674G occurred only in the Black and Hispanic groups. 3′-UTR T+601C was an unusual variant in Hispanics, while it was substantially more common in the other three groups.

Although A-585T, [ATTT/−] –464 Ins/Del, and A+8344G (within the 3′-UTR; A+1050G) were common in all four ethnic groups, their frequencies differed among the groups.

Linkage disequilibrium (LD) across the *NPY1R* locus

Pairwise LD among four common SNPs and the Ins/Del across *NPY1R* was qualified as parameter r^2, scaled from 0 to 1. On-line Figure 1 shows the LD and haplotype block structure in four 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 four common polymorphisms were located within a single LD block (by the 4 gamete rule in Haploview), while 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±7.3%, P<0.0001), initial DBP (h^2=28.7±8.6%, P=0.001), final SBP (h^2=28.3±7.9%, P=0.0004), final DBP (h^2=30.1±7.8%, P=0.0001), DBP change (h^2=26.7±8.5%, P=0.0014), and SBP change (h^2=19.8±9.7%, P=0.023). Heritability was also significant for baroreceptor coupling in the low frequency domain (0.05-0.15 Hz; h^2=52.6±5.9%, P<0.0001), high frequency domain (0.15-0.4 Hz; h^2=41.2±7.5%, P<0.0001), and for baroreceptor slope, both upward deflections (h^2=25.8±10.5%, P=0.009), and downward deflections (h^2=38.6±7.2%, P<0.0001).

NPY1R variant association with autonomic traits in twins

We began by testing whether *NPY1R* genetic variation affected physiological traits in twins. Since the number of LD blocks was limited (on-line Figure 1), we chose two common SNPs to “tag” the locus: promoter/A-585T and 3′-UTR/A+1050G.

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 (0.15-0.40 Hz, p=0.015) and low frequency (0.05-0.15 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 (12). Significant prediction by A +1050G was observed for not only final (post-cold) DBP (P=0.0095), but also for the 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. 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 BMI in twins (for promoter/A-585T, p=0.81; for 3′-UTR/A+1050G, p=0.74).

NPY1R polymorphisms and hypertension

3′-UTR A+1050G and promoter A-585T were scored in n=936 individuals selected from the extreme upper/lower 5th %iles of BP in a large primary care population (on-line Table 2). Each SNP showed significant effects on both DBP (Figure 3, A-585T, p=1.2E-4; A+1050G, p=0.001) and SBP (A-585T, p=0.018; A+1050G, p=0.012).

In addition, there was a 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 versus low), using both 2-way ANOVA and regression. On ANOVA, BP status was influenced by promoter A-585T (p=3.92E-4), as well as 3′-UTR A+1050G (p=0.005), and the interaction of the two (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 (Figure 4): subjects with two copies of Hap-4 displayed increases in both SBP and DBP (SBP, p=0.007; DBP, p=2.34E-5). When BP was analyzed as a dichotomous trait (Fisher's Exact Test), Hap-4 copy number affected the trait (p=2.3E-4).

Although the hypertensive subjects displayed higher BMI than the normotensive group (30.6 ±0.3 versus 24.6±0.2 kg/m^2, p<0.001; on-line Table 2), neither of the two NPY1R variants associated with BMI in this sample (promoter/A-585T, p=0.096; 3′-UTR/A+1050G, p=0.918).

Statistics: Alpha threshold

We reevaluated the alpha threshold required to avoid false-positive conclusions in the setting of two genetic markers (3′-UTR A+1050G and promoter A-585T) by using SNPSpD (SNP SPectral Decomposition) (13), 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 two 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, Figure 5A); the A>G difference was also seen embryonic kidney cells (on-line Figure 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 (r^2=1) with the −464[ATTT] Ins/Del in the white population: the A-585 allele appeared consistently with −464ATTT Ins, while 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 transfection into PC12 cells for 24 hours, luciferase activity was measured.

The promoter haplotypes displayed significantly different luciferase activities ($p<0.001$, Figure 5B). Between the two 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 (Figure 5B): on a background of the −464 Del allele, −585T was more active 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 (on-line Figures 3A and 3B). Based on the chimpanzee sequence, the likely human ancestral allele at A+1050G is A (the most frequent allele), while at A-585T the likely ancestral allele is A (also the most frequent allele). While the promoter region spanning A-585T was well-conserved, no clearcut 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 (Figure 6). 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 (Figure 5A).

DISCUSSION

Overview

In this study, we probed whether common genetic variation at the NPY vasoconstrictive receptor (*NPY1R*) locus might influence autonomic function and disease. We began by undertaking systematic polymorphisms discovery at *NPY1R*, revealing several novel variants in likely functional domains, especially non-coding 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 post-transcriptional 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(14). NPY1R is the widely expressed major subtype of NPY receptor, where it mediates *vasoconstriction* in response to peripheral NPY (15). In the CNS, studies of an NPY transgenic rat by Mielzyslaw 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 towards catecholamine secretion. In *NPY1R* knockout mice they also found increases in adrenal catecholamine content, constitutive catecholamine release, and plasma catecholamine concentrations. In Y_1 receptor-expressing cells, NPY decreased the activity of tyrosine hydroxylase (TH), 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 three indices; 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 such pressor responses may eventuate in sustained/fixed BP elevation (12). Thus both baroreceptor dysfunction and increased BP pressor responses may be risk indicators for future development of hypertension (1,12). Of note for the Y_1 receptor, *NPY1R* knockout mice display hyperalgesia to thermal, cutaneous, and visceral chemical stimuli (14), though their pressor responses to cold stimulation are reportedly unchanged (16).

Finally, our genetic results suggest a role for a specific variant, *NPY1R* A+1050G, in triggering a pleiotropic series of effects on cardiovascular risk (Figures 2,3,4); 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 (Figure 2), suggesting that the G (minor) allele may act recessively in this regard. The substantial frequency of the G (minor) allele (at 25-50%; on-line Table 1) allowed us to detect the G/G effect in sufficiently large population samples (here: 376 twins and sibs).

Hypertension: Genetic interaction

In subjects with the most extreme BPs in the population (Figure 3), promoter A-585T and 3′-UTR A+1050G each had substantial effects upon both DBP and SBP. In addition, we found that these two 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 (Figure 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 mmHg SBP/DBP) in individuals who bore two 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 (17). In the human *NPY1R* transcript, bioinformatic analyses indicated that variant A+1050G is located in a motif recognized by hsa-miR-511 (Figure 6). 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 (17). We recently characterized functional variants that contribute to hypertension risk in the 3′-UTRs of *CHGA* (chromogranin A; a protein catalytic in formation of catecholamine storage vesicles (12) and *GCH1* (GTP cyclohydrolase; rate-limiting in formation of pterin cofactors) (18).

Promoter—In the promoter, common variants A-585T and Ins/Del/-464 each demonstrated functional activity in luciferase reporter constructs (Figure 5B). Hypertension-associated promoter allele -585T decreased promoter expression, whether studied in isolation (Figure 3) or in the context of its naturally occurring haplotype (Figure 4). Although the local sequence region around A-585T is conserved across species (on-line Figure 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 (19). Multiple autonomic phenotypes were measured in the twins, which permitted estimation of trait heritability as well as defining the 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* (Figures 5A and 5B, on-line Figure 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 (Figure 4).

Caveats—First, although we conducted systematic polymorphism discovery in four 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 (Figure 4) without multiple testing. Second, we applied SNPSpD(13) 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 two independent samples (twin pairs and population BP extremes). Ultimately, our mechanistic studies *in cella* (Figures 5A and 5B, on-line Figure 2) lend plausibility to our results.

Finally, while we found that *NPY1R* variants which 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 whereby the central effects of *NPY1R* polymorphism predominate on trait determination (Figure 7). However, while 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 (Figure 1). Such genetic variation, especially in the 3′-UTR, predicts inter-individual variability in early, pathogenic autonomic traits that are precursors to development of hypertension, such as baroreceptor dysfunction and exaggerated pressor responses to stress (Figure 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 (Figure 7) 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.

METHODS

Subjects and clinical characterization

Polymorphism discovery and biogeographic ancestry—Initially, a series of *n*=80 individuals (2n=160 chromosomes) was studied by resequencing of *NPY1R* for systematic polymorphism discovery: *n*=20 of European ancestry (white), *n*=20 sub-Saharan African Ancestry (black), n=20 Asian ancestry and n=20 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(20). Subjects were volunteers from southern California, and each subject gave informed, written consent; the protocol was approved by the UCSD 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(21;22) as well as by newspaper advertisement(21). These twin individuals were all of European ancestry, to permit allelic association studies within 1 ethnicity. There were *n*=360 twin individuals from *n*=180 white (European ancestry) twin pairs, including *n*=125 monozygotic pairs and *n*=55 dizygotic (DZ) pairs. The twin/sibling subjects were 14-84 years old.

Hypertension—We studied n=936 (n=404 male, n=532 female) white (European ancestry) subjects, recruited from a large primary care (Kaiser Permanente) population in San Diego, as previously described (23). In this primary care population, ~81% attended the clinic, and ~46% consented to participation in the study, with collection of blood for preparation of genomic DNA. From consented participants, the subjects in this study were selected, based upon measurement of DBP, to represent the highest and lowest ~5th DBP percentiles in that population. n=426 subjects were chosen for higher DBPs, while n=510 subjects were chosen for lower DBPs. The DBP criterion was chosen because of its heritability (21). The statistical power of association between biallelic DNA markers and human quantitative trait loci can be substantially augmented by the sampling individuals from opposite (upper and lower) ends of the trait distribution (24,25,26)and analyses of the quantitative trait in extreme subjects (as opposed to dichotomization of the trait) further enhances power (27). 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 the females. To accomplish this, lower BP individuals (SBP/DBP 108±0.7/56±0.2 mmHg) were selected from the bottom 4.8th percentiles of DBP, while the higher BP group

(SBP/DBP 154±0.8/99±0.3 mmHg) 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 (28), the very large, epidemiological scale of this primary care-based ascertainment precluded its use here. Forty-one percent of patients in the higher BP group were taking one or more antihypertensive medications (including 15% on diuretics and 19% on angiotensin-converting enzyme [ACE] inhibitors), while none 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 mmHg) to each treated BP, as described (29). Although such adjustments are necessarily imperfect, their value is reinforced by restoration of familial (sibling/sibling) BP correlations (29); alternative adjustments based on specific medication categories (30) were not used.

Genomics

Systematic polymorphism discovery by resequencing—Genomic DNA was prepared from leukocytes in EDTA-anticoagulated blood, using PureGene extraction columns (Gentra; Qiagen) as described previously(31). Public draft human genome sequences were obtained from the UCSC Genome Bioinformatics website (http://genome.ucsc.edu) and used as a scaffold for primer design. The base position numbers were from NCBI *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 PCR 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 PCR from 20 ng genomic DNA in a final volume of 25 ul, which also contained 0.1 U of Taq DNA polymerase (Applied Biosystems), 200 nM of each dNTP, 300 nM of each primer, 50 mM KCl, and 2 mM $MgCl_2$. PCR was performed in an MJ PTC-225 thermal cycler, starting with 12 minutes of denaturation at 95°C, followed by 45 cycles at 95° C for 30 seconds, 63°C for 1 minute (annealing), and 72°C (extension) for 1 minute, and then a final extension of 8 minutes at 72°C. PCR products were treated with exonuclease I and shrimp alkaline phosphatase to remove primers and then dNTPs prior to cycle sequencing with BigDye terminators (Applied Biosystems). Sequence was determined on an ABI-3100 automated sequencer. Polymorphism and heterozygosity were detected using CodonCode Aligner <http://www.codoncode.com/>.

SNP genotyping—Genotypes were determined by two extension-based techniques, either the MALDI (matrix assisted laser desorption ionization) mass spectrometry method of Sequenom, or the luminescent base incorporation method of Pyrosequencing (Biotage), 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 (32). To probe autonomic control of the circulation in twin pairs, BP and HR were recorded continuously and non-invasively for 5 minutes in seated, resting subjects with a radial artery applanation device as well as thoracic EKG electrodes, and dedicated sensor hardware (Colin Pilot; Colin Instruments) and software (ATLAS, WR

Medical Electronics Co; and Autonomic Nervous System, Tonometric Data Analysis [ANS-TDA], Colin Instruments). Baroreceptor slope and heart rate variability (HRV) were quantified as previously described (33). The BP and HR responses to environmental (cold) stress (one hand immersed in ice water for one minute) were obtained with the same hardware and software, as previously described (33).

NPY1R variants (promoter A-585T, and 3′-UTR A+1050G): Functional consequences *in cella*

3′-UTR—The 1338 bp 3′-UTR was amplified with PCR primers incorporating XbaI restriction sites, facilitating ligation into the unique XbaI site in the luciferase reporter plasmid pGL3-Promoter (Promega); 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 PCR 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). 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 pCMV-beta-Gal, encoding the beta-galactosidase reporter plasmid under control of the CMV promoter (Promega). After transfection and cell growth over an 8- to 24-hour 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 (GEEs; PROC GENMOD) in SAS (Statistical Analysis System). Population BP extreme analyses were performed by 2-way ANOVA in SPSS (Statistical Package for the Social Sciences).

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(34). This method maximizes the likelihood of the estimate assuming a multivariate normal distribution of phenotypes in twin pairs (MZ versus DZ) 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 SOLAR, 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 (35), 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, available at <http://research.calit2.net/hap> (36).

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 one SNP within an LD block was associated with a trait, SNPSpD (13) 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 ANOVA in SPSS. Diastolic BP value was taken as the dependent variable, while 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/>. RNA hybrid structures and predicted minimum folding energies were analyzed at <http://bibiserv.techfak.unibielefeld.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 mRNA stem/loop structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support: Department of Veterans Affairs, National Institutes of Health. We appreciate the support of NIH/NHLBI (HL58120), the NIH/NCMHD-sponsored (MD000220) EXPORT/CRCHD minority health center, as well as the NIH/NCRR-sponsored (RR00827) General Clinical Research Center.

ABBREVIATIONS

BP	Blood pressure.
DBP	Diastolic blood pressure.
Del	Deletion.
CNS	Central nervous system.
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.

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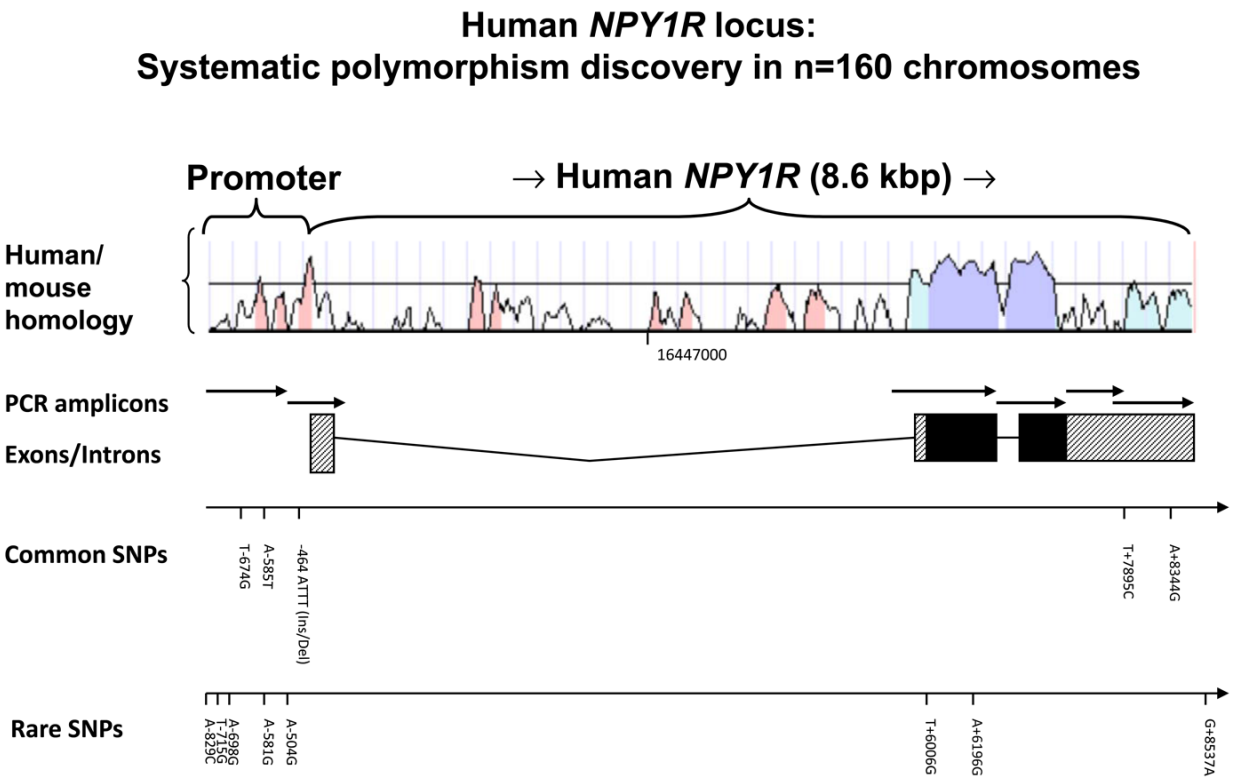

Figure 1. NPY1R resequencing strategy and identified variants

Sequences conserved between mouse and human *NPY1R* were visualized with VISTA <http://genome.lbl.gov/vista/index.shtml>. Locations of common (minor allele frequency >5%) and rare SNPs are indicated. Positions are numbered upstream or downstream of the CAP site. Solid blocks: Open Reading Frame. Hatched bocks: UTRs.

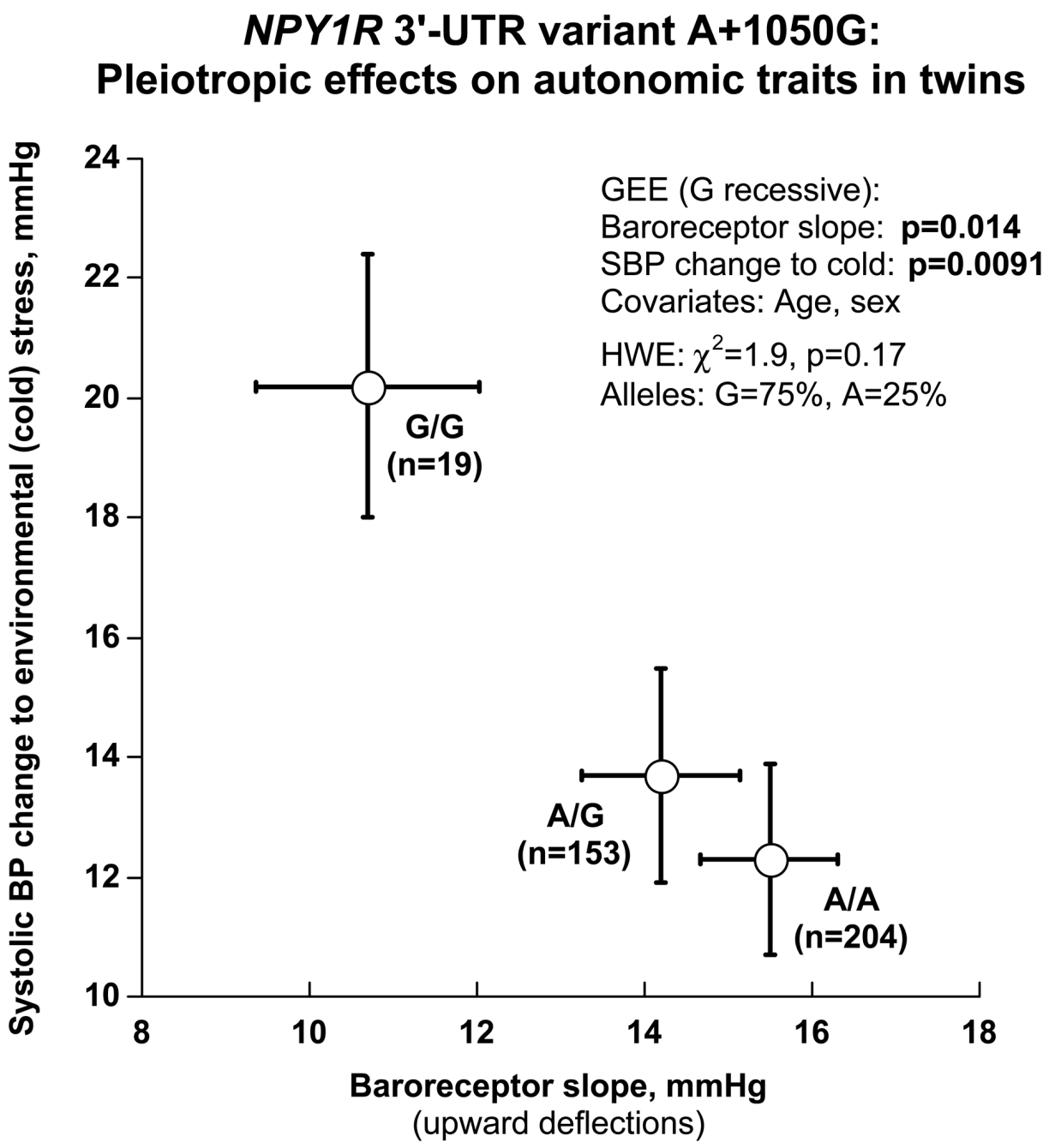

Figure 2.
Pleiotropic effects of *NPY1R* 3′-UTR variant A+1050G on coupling between baroreceptor function and the BP change in response to environmental (cold) stress.

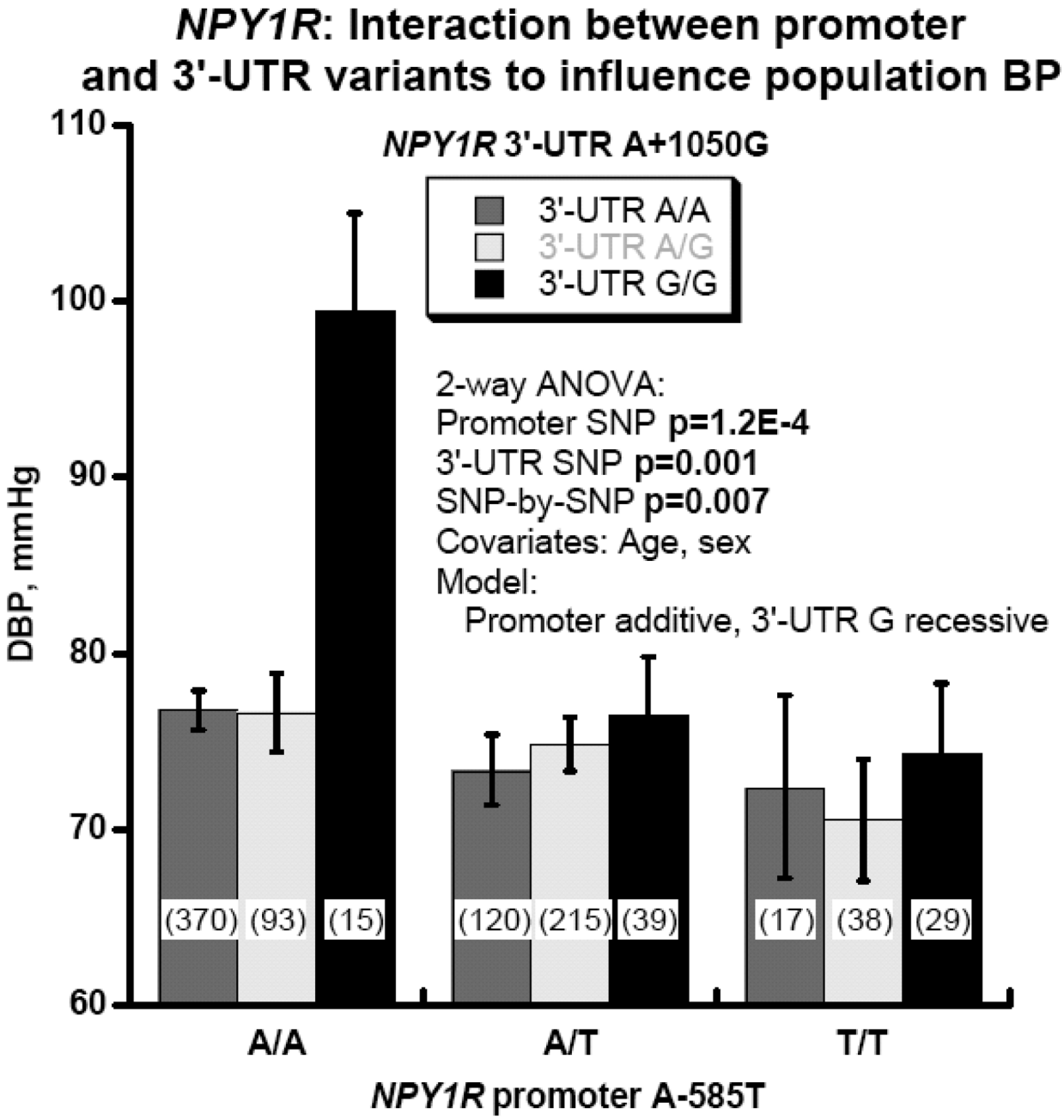

Figure 3.
NPY1R common variants in the 3′-UTR (A+1050G) and proximal promoter (A-585T) influence BP both independently and interactively.

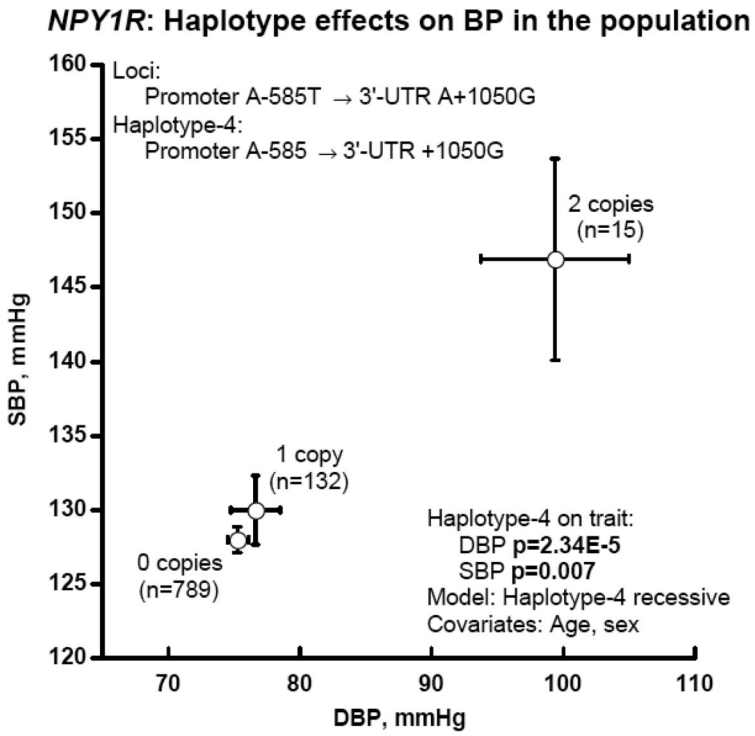

Figure 4.
NPY1R haplotype 4 (A-585 → +1050G) is associated with both DBP and SBP in a European ancestry population.

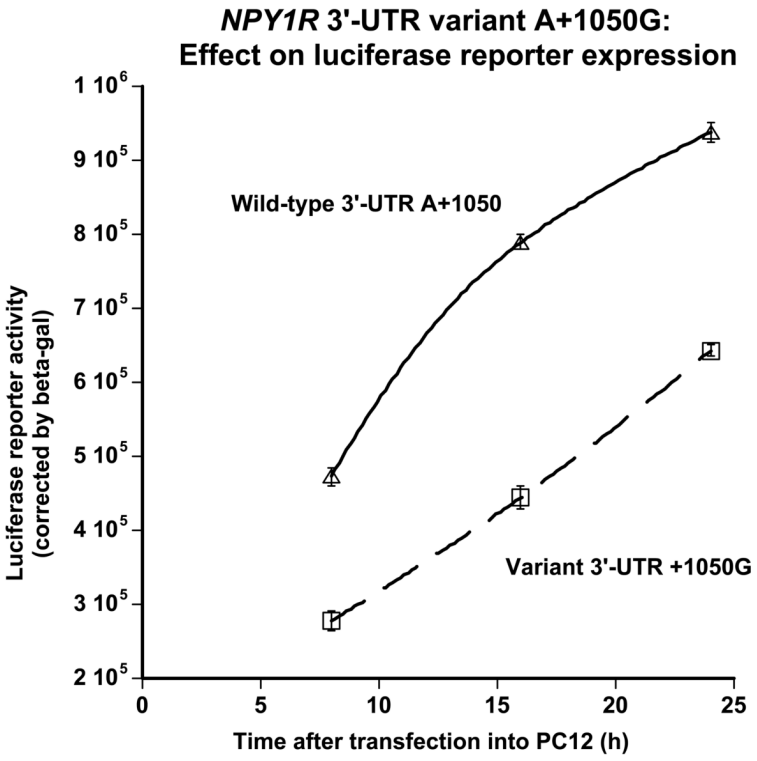

Figure 5A. ***NPY1R*** **3′-UTR polymorphism A+1050G influences gene expression** ***in cella***
Results of luciferase reporter/3′-UTR variant transfections into PC12 cells are shown.

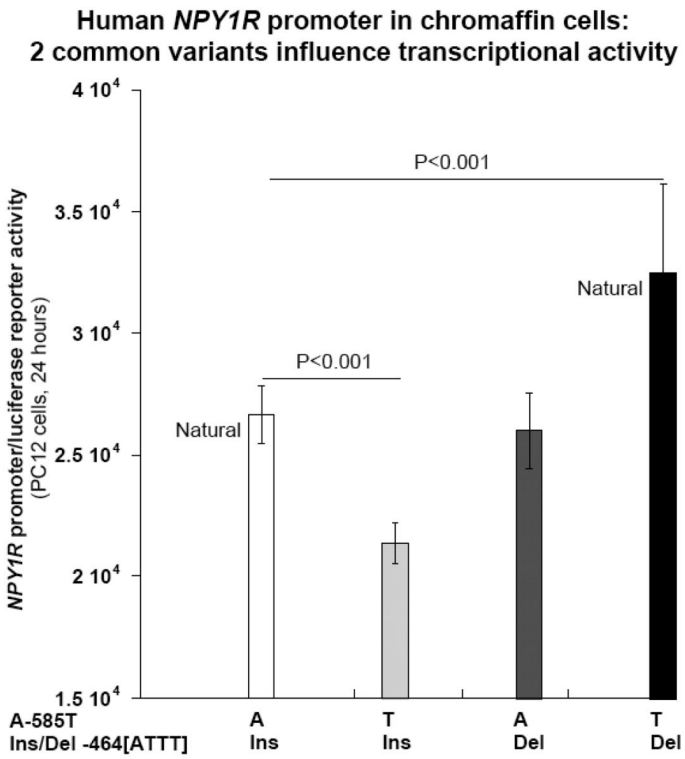

Figure 5B. Effect of *NPY1R* common promoter variants (A-585T and Ins/Del) on gene expression *in cella*

Natural: Haplotype combination found in human *NPY1R* promoter DNA.

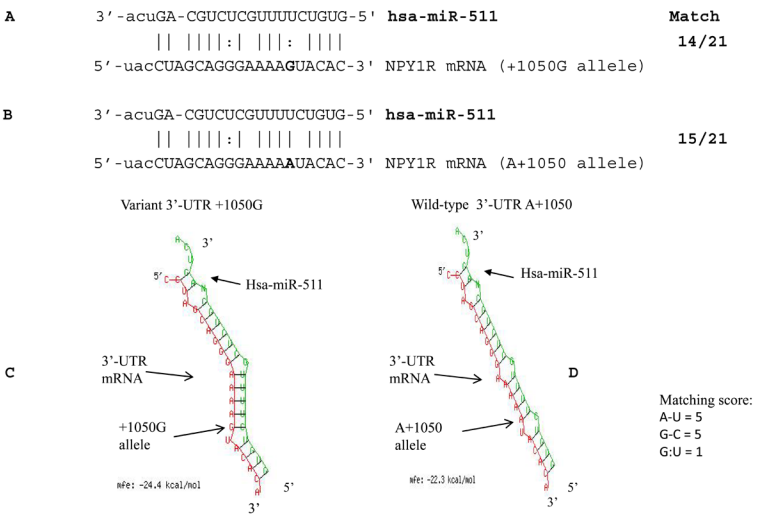

Figure 6. ***NPY1R*** **3′-UTR variant A+1050G occurs in a micro-RNA recognition motif**

A, B. Complementarity between hsa-miR-511 and the *NPY1R* 3′-UTR site (1034–1055 bp downstream from the *NPY1R* stop codon), variant (panel **A**) versus wild-type (panel **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 (panel **D**) or G (panel **C**) alleles at A+1050G. Arrows highlight the polymorphic site. Calculated mfe: minimum free energy.

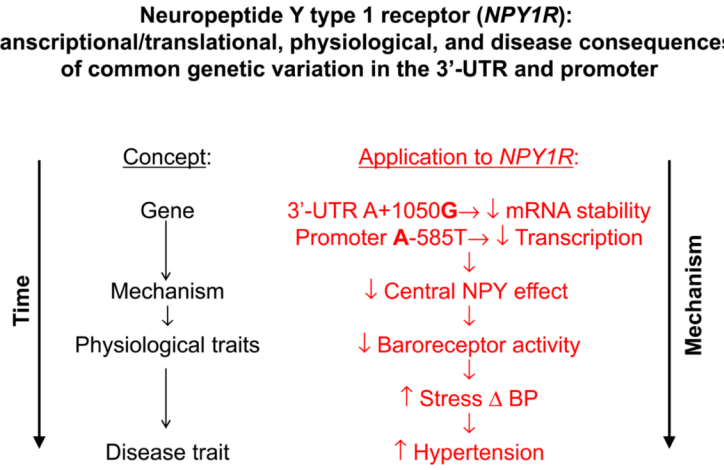

Figure 7. Neuropeptide Y type 1 receptor (*NPY1R*)
Transcriptional/translational, physiological, and disease consequences of common genetic variation in the 3′-UTR and promoter

Table 1

Heritability (h^2) and effects of *NPY1R* 3′-UTR variant A+1050G on physiological traits in twins siblings pairs

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 n=360 twins. Values are age and sex adjusted in SOLAR, and expressed as %. P value is the significance of the heritability value. Single nucleotide polymorphism (SNP) statistics for twin & sibling study population were derived from generalized estimating equations (GEEs; PROC GENMOD) in SAS, to account for correlations within families. Bold type: p<0.05.

	Heritability (h^2) (n=360 twins)		***NPY1R* 3′-UTR A+1050G diploid genotype (n=376 twins and sibs)**			
Phenotype	**% ±SEM**	***P***	**A/A or A/G (n=357)**	**G/G (n=19)**	**χ^2**	***P***
Baroreceptor coupling (frequency domain), msec/mmHg						
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), msec/mmHg						
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 (mmHg)						
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**