Online Appendix for the following *JACC* article

TITLE: Neuropeptide Y (NPY)

Genetic Variation in the Human Promoter Alters Glucocorticoid Signaling, Yielding Increased <CASE>NPY</CASE> Secretion and Stress Responses

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APPENDIX

SUPPLEMENT.

Introduction

Previous studies have associated genetic variation at the *NPY* locus with human disease traits: ischemic stroke (promoter C-399T, rs16147)(1,2), cholesterol metabolism (Leu7Pro)(3,4), retinopathy in type II diabetics (Leu7Pro) (5) (6), type II diabetes itself (Leu7Pro) (7) (8) , cardiovascular disease and high blood pressure (Leu7Pro)(9-11), alcohol dependence (Leu7Pro)(12), obesity (rs3037354, promoter ∇-880Δ, rs3037354)(13), schizophrenia (C-399T)(14), brain responses to stress and emotional challenge (C-399T)(15), and early-onset atherosclerosis (C-399T)(16). In addition, genome-wide linkage analysis of pulse pressure in native Americans revealed a QTL peak harboring *NPY* within the confidence interval (17). In rodent genetic models of human disease <http://rgd.mcw.edu>, the *Npy* locus lies within the confidence intervals for linkage to blood pressure (Bp21, Bp24, Bp79, Bp86) and heart rate (Hrtrt6, Hrtrt13).

Methods

Subjects and clinical characterization.

Polymorphism determination. Since allele frequencies and haplotypes may differ substantially across biogeographic ancestries, a series of n=80 individuals (i.e., 2n=160 chromosomes, supplementary Table 1) was selected to span a diverse range of 4 biogeographic ancestry groups: white (European ancestry), black (sub-Saharan African), east Asian, and Hispanic (Mexican American) ethnicities, for systematic/comprehensive polymorphism determination by re-sequencing. Characterization of 2n=160 chromosomes afforded us >99% power for detection of polymorphisms with as low as ~2.8% minor allele frequency. Ethnicity was established by self-identification. None of the subjects had a history of secondary hypertension, renal failure or diabetes mellitus.

NPY replication in young American men. US Marines from the MRS (Marine Resilience Study) (18) <http://marineresilience.org/>. N=361 Marines were recruited at Marine Corps bases in Southern California. They were all healthy males of self-identified European (white, non-Hispanic) ancestry, ranging in age from 18-37 years (mean 21.7±2.5 years). Biological samples including whole blood (for genomic DNA) and EDTA plasma (for NPY) were collected by venipuncture for further analysis.

NPY replication in Australia twins. The characteristics of the QIMR twins (zygosity, age, sex, biogeographic ancestry) have been described previously by us (19). Here we studied plasma NPY in n=2212 twins, both MZ and DZ. QIMR twins were then probed by a genome wide association study, using Illumina 610K, 317K or 370K chips, with imputation to non-typed HapMap SNPs, as described (20).

Molecular biology

Systematic polymorphism determination at the *NPY* locus. Public draft human (21) and mouse (22) genome sequence was obtained from the UCSC Genome Bioinformatics website (http://genome.ucsc.edu) and used as a scaffold for primer design and sequence alignment. Positions were numbered with respect to the mRNA cap (transcriptional initiation) site in NCBI RefSeq source clone NM_000905.2. PCR primers we designed by Primer3 (23) to span each of the 4 exons, as well as including 50-100 bp of flanking intronic sequence, and 1382 bp of proximal promoter (upstream of the cap site). The primers are shown in supplementary Table 2. PCR, amplicon purification, sequencing (on an ABI-3100 capillary device) and analysis of target sequences were performed according to the protocol described previously (24). Rare (<5% minor allele frequency) SNPs were confirmed by re-sequencing in multiple individuals, or from the reverse direction.

Genotyping of *NPY* variants . SNP diploid genotypes at *NPY* were scored either by a one base extension system in the ABI PRISM® SNaPshot™ multiplex kit (Cat#4323161; Foster City, CA) using an ABI-3130 capillary sequencer, or by Pyrosequencing (Biotage, Sweden). PCR, SNaPshot and Pyrosequencing primers are shown in supplementary Tables 3 and 4.

Genotyping of *NPY* promoter variant ∇-880∆. An AFLP (Amplified Fragment Length Polymorphism) was used to genotype the 2-bp promoter Ins/Del (rs3037354) with forward primer 5’-aggtttaacgcgatgagcaa-3’, and reverse primer 5’-acatgagtgtggcgactcaa-3’. After PCR over 30 cycles and digestion with the enzyme AlwN1 (1 unit for 4 hours), 2% agarose gel electrophoresis separated restriction fragments corresponding to the original alleles. The Del/Del genotype yielded a single 300-bp band; the Ins/Ins genotype two shorter bands (170 bp and 130 bp); while Ins/Del heterozygotes displayed 3 bands (300 bp, 170 bp and 130 bp).

Computation

Genome-wide linkage analysis. Multipoint linkage (meiotic co-segregation) analysis of plasma NPY concentration was carried out with SOLAR (Sequential Oligogenic Linkage Analysis Routines)(25), available at <http://txbiomed.org/departments/genetics>. This analysis included 730 autosomal markers in 228 individuals derived from twin and sibling pairs (26). Linkage was assessed by fitting a polygenic model that did not incorporate genotype information provided by marker loci, and comparing it with models that incorporate genotype data with multiple markers. The log (base 10) of the ratio of the likelihoods of the marker-specific and polygenic models is the log of the odds (LOD) score, a traditional measure of genetic linkage. Positional candidate loci beneath the *NPY* LOD peak on chromosome 7p15 were identified, with respect to the microsatellite marker positions (in cM), using the integrated genetic/physical distance maps of Duffy (27), viewed at <http://genepi.qimr.edu.au/staff/davidD/>.

Data transformation. Plasma NPY values did not display excessive kurtosis or skewness. During marker-on-trait associations *in vivo*, results of descriptive statistics (mean±SEM for each diploid genotype) were plotted in order to select the optimal model (additive versus dominant/recessive) for inferential statistical test (F, chi-square, p). To adjust for effects of treatment in hypertensives, blood pressure values were transformed by adding by 10/5 mmHg for those on anti-hypertensive drugs (28).

Heritability of phenotype expression *in vivo*. In twin pairs, heritability (or the fraction of trait variance accounted for by genetic variance, $h^2=V_G/V_P$) was estimated by SOLAR (Sequential Oligogenic Linkage Routines)(25), available at <http://txbiomed.org/departments/genetics>.

Haplotypes and linkage disequilibrium (LD). Haplotypes were inferred from common single nucleotide polymorphisms (∇-880Δ, C -399T, C1000G, G1201A and C5327T; minor allele frequencies ≥20%), and the less common SNP T1071C (rs16139, Leu7Pro) in the signal peptide of *NPY,* by the HAP algorithm (29). Pair-wise linkage disequilibrium (LD) between these SNPs was visualized as R^2 using the solid spline method in Haploview (30).

Marker-on-trait association. During associations at SNPs (or their haplotypes), subjects were categorized according to either diploid genotype at a bi-allelic SNP locus, or carrier status (2, 1, or 0 copies) for a particular haplotype. We began with *NPY* haplotype tests, to avoid multiple comparisons during the initial stage of genetic association; nonetheless, SNP spectral decomposition (SNPSpD, at <http://gump.qimr.edu.au/general/daleN/SNPSpD/>) indicated that the 5 common SNPs across NPY were sufficiently highly correlated to yield an "effective number" of SNPs at only 3; thus a new target alpha=0.017 was sufficient to maintain the experiment-wide alpha at 0.05. In consideration of testing the effects of one genetic variant on multiple correlated traits, we employed estimation of the FDR (False Discovery Rate), in order to minimize false negative results while maximizing false positive results, using the Excel calculator of FDRs from p-values, at <http://www.rowett.ac.uk/~gwh/fdr.html>.

Promoter motif analyses. Transcription factor binding sites were identified from position weight matrices at the TRANSFAC database <http://www.gene-regulation.com>, using the platform MAPPER at <http://mapper.chip.org> (31), and the motifs were confirmed and visualized by JASPAR at <http://jaspar.genereg.net/> (32).

Human twin phenotyping

Biochemical. *Plasma NPY measurement* . EDTA-anticoagulated plasma in UCSD twins/siblings was obtained from an antecubital vein in seated subjects, and stored at -70°C prior to assay. Plasma NPY was measured by radioimmunoassay (RIA) based on synthetic human [^{125}I]-NPY_{1-36}-amide, as previously described (33) with reagents from Eurodiagnostica (Malmo, Sweden). The assay sensitivity (lower limit of detection) was 6 pmol/L. In this assay, the molar cross-reactivities of related family peptides (human peptide YY, pancreatic polypeptide, NPY_{1-21} and NPY_{20-36}) were all <2%. The intra-assay coefficient of variation was 2.6-3.9%, while the inter-assay coefficient was 10.5-12.7%. Mean recovery of exogenous NPY added to plasma was 82% in this system.

Plasma NPY in Australia twins was measured by a fluorescent enzyme immunoassay (FIA) protocol using biotinylated NPY, with reagents from Phoenix Pharmaceuticals (Burlingame, CA); the intra-assay coefficient of variation was 1.6-7.6%, with a lower limit of detection of ~1 pg/ml (or ~0.23 pmol/L). NPY_{3-36} cross-reacted by 14%, while other peptides of that family (PYY, PP) did not cross-react. The inter-assay (RIA versus EIA) correlation on the same samples was r=0.77, p=0.0008.

Physiological (*in vivo*).

Cardiac and vascular function at rest. Baseline/resting cardiovascular traits (SBP, DBP, HR, CI [cardiac index], and SVRI [systemic vascular resistance index]) were estimated non-invasively using the DynaPulse oscillometric device (PulseMetric, Vista, CA) as previously described (34). Flow parameters (e.g., CO, SVR) were normalized to body surface area, to yield CI and SVRI. The correspondence between DynaPulse and invasive determinations of cardiac output and systemic vascular resistance has been documented recently (35).

Environmental (cold) stress test. To probe the functional significance of common variation at *NPY*, we examined the potential association of 5 common *NPY* polymorphisms with the blood pressure response during the environmental (cold) stress test (36) on 399 twin (MZ or DZ) and sibling individuals. During the stressor, the subject immersed the non-dominant hand into ice (0°C) water for one minute, with averaged measurements of SBP, DBP, and HR, stable over 3 beats pre- and post-procedure.

***NPY* promoter/luciferase reporter activity assays**. Haplotype-specific promoter fragments corresponding to *NPY* −1006/+86 bp were PCR-amplified from genomic DNA of known homozygotes, and cloned into promoter-less firefly luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI). Promoter mutants and haplotypes were created, if necessary, by site-directed mutagenesis (QuikChange, Stratagene), and verified by dideoxy sequencing (supplementary Figure 1). PC12 pheochromocytoma cells were transfected (at 50-60% confluence in 15.6 mm polystyrene dishes) with 500 ng of supercoiled promoter haplotype-firefly luciferase reporter plasmid by the liposome method (Transfectin; Bio-Rad, Hercules, CA). The firefly luciferase activity and protein in the cell lysates were measured 24 hours after transfection, and the results were expressed as the ratio of firefly luciferase/total protein, as described previously (37,38). Each experiment was repeated a minimum of four times. Results were expressed as mean ± SEM. Statistical significance ($p<0.05$) was calculated using the student t-test or ANOVA, as appropriate.

Effect of glucocorticoid and PAX6 on *NPY* promoter/luciferase reporter activity: In order to remove the effect of possible residual glucocorticoid hormone, we used charcoal/dextran-stripped fetal bovine serum (Gemini, cat: 100-119; West Sacramento, CA 95605) instead of regular FBS. The glucocorticoid agonist dexamethasone (cat#: D4902) was purchased from Sigma-Aldrich (St. Louis, MO). The full-length human PAX6 cDNA in a pCMV eukaryotic expression plasmid (pCMV6-XL5) was purchased from Origene (cat#: SC127933, Rockville, MD).

Electrophoretic mobility shift assays (EMSA).

Preparation of nuclear extracts. PC12 chromaffin cells were grown in 10-cm diameter tissue culture plates and nuclear extracts were prepared using a commercial kit (cat#: 10009277; Cayman Chemical, Ann Arbor, Michigan), as described. Protein concentrations were measured by the Bio-Rad coomassie blue dye-binding reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Synthesis and labeling of oligonucleotides. Single-stranded oligodeoxynucleotides flanking the sequences of *NPY* promoter ∇-880Δ or C-399T, and their complementary (-) strands, were synthesized and PAGE-purified by Valugene (San Diego, CA), and used at a concentration of 100 μM. The sequences are shown as on-line supplementary Table 6. 13-16 additional flanking bases were incorporated on each side of the variant. Five pmol of each oligonucleotide were labeled with biotin by a Biotin 3'-End DNA Labeling kit (Cat 89818; Pierce, Rockford, IL), as described. Annealing of oligonucleotides was accomplished by mixing together equal amounts of labeled complementary strands, and incubating the mixture for 1 hour at room temperature.

Electrophoresis. Binding was studied by LightShift chemiluminescent EMSA kit (cat#: 20148; Pierce, Rockford, IL) in 15 μlreaction complexes; i.e., 1x binding buffer, 100 ng/μl poly-(dI.dC), with or without unlabeled (competitor) target DNA oligonucleotide (10 pmol), and nuclear protein extract (4 μg), with or without anti-factor antibodies (4 μg), and biotin-labeled oligonucleotide (0.05 pmol). Anti-GR antibody was purchased from Santa Cruz biotechnology (cat#: sc-8992 X; Santa Cruz, CA). After incubating for 20 min at room temperature, the mixtures were loaded onto 5% non-denaturing polyacrylamide gels (5% acrylamide, 37.5:1 acrylamide:bisacrylamide) that were run at 100 volts in 0.5X TBE buffer. The gels were transferred onto nylon membranes (cat#: 77016; Pierce, Rockford, IL), and biotin-labeled DNA was detected by chemiluminescence (Pierce).

Supplementary Figure 1. Human *NPY* promoter reporter plasmid construction. The 1506-bp (-1006 to +86 bp) *NPY* promoter fragments were amplified from human genomic DNA and subcloned directionally (5'→3') into unique polylinker Nhe I and Hind III restriction sites of a firefly luciferase reporter vector (pGL3-Basic, Promega, Madison, WI). Haplotypes in each insert were verified by dideoxy sequencing before further use.

Supplementary Figure 2: Genetic variation at the human *NPY* locus. **LD blocks across the locus in 4 biogeographic ancestry groups**. Common (minor allele frequency >20%) SNPs were used to demonstrate LD (numbering from the cap site): rs3037354 (Ins-880Del), rs16147 (C-399T), rs16140 (C1000G), rs5573 (A1201G), and rs5574 (C5327T). LD blocks were assigned by the solid spline method in Haploview. The *NPY* exon/intron structure is superimposed above each panel; there were no common variants in the final (most 3') exon.

Supplementary Figure 3. *NPY* promoter C-399T polymorphism: *PAX6* motif.

Left panel: Inter-species conservation, and consensus motifs match for PAX6 at C-399T. Sequence alignments were done by Clustal-W, while motifs from TRANSFAC were explored at Chip-Mapper and JASPAR (see computational Methods).

Right panel: Functional response: Effect of PAX6 *trans*-activation on *NPY* promoter activity in PC12 cells. 50 ng of human PAX6 expression plasmid (in a pCMV vector) were co-transfected with 500 ng of each of the 4 *NPY* promoter haplotype/reporter plasmids. Cells were harvested at 24 hours after co-transfection. Co-transfection of PAX6 substantially increased NPY gene expression (p=1.88E-07), but (even in the face of a better PAX6 match for the C (9/14 bp) than T (8/14 bp) alleles) the effects of PAX6 were inconsistent on C-399T: increasing reporter expression by ~2.52-fold (p=0.002) on the ∇-C haplotype, but ~0.54-fold (p=0.007) on the Δ-T haplotype. Little effect was observed on ∇-T or D-C genotypes (supplementary Figure 3, right panel). Thus, the effect of PAX6 on the C-399 allele is either inconsistent, or quite dependent upon haplotypic background (i.e., only seen with the -880∇ allele).

Supplementary Figure 4. *NPY* C-399T polymorphism and *PAX6* motif: EMSA (Electrophoretic Mobility Shift Assay) to probe interaction of C-399T and the synthetic *PAX6* element with chromaffin cell nuclear proteins. Biotin-labeled versions of both the C and T alleles are shown, and could be shifted by chromaffin cell nuclear proteins, but the shifted bands could be displaced by unlabeled versions of either C or T, weighing against specificity of binding.

Supplementary Figure 5: Transfected *NPY* promoter/luciferase reporter activity: Haplotype and allele effects. Differential luciferase reporter activity is shown in transfected pheochromocytoma (PC12) cells. Six replicate transfections were done for each haplotype. General linear model (ANOVA) tests (with reporter activity as the dependent variable) were carried out for either the 4 haplotypes, or the two diploid genotypes in the promoter. The frequencies of the four 2-SNP-derived (∇-880Δ→C-399T) promoter haplotypes in the general population (here, the blood pressure extremes) were as follows: ∇-C, 52.8%; Δ–T, 25.8%; ∇-T, 21.3%; and Δ–C, 0.04%. At baseline, C-399T variation had no effect the gene expression at either ∇-880 or -880D backgrounds (p=0.83).

Supplementary Figure 6. *NPY* promoter variant ∇-880Δ: Coordinate directional function *in cella* and *in vivo*. *In cella* results (horizontal axis) are obtained from *NPY* promoter-transfected PC12 cells, with ∇-880Δ allelic variation on a background of the C-399 allele (Figure 5). *In vivo* results (vertical axis) are from twins/siblings (Figure 3B), with results are grouped by presence or absence of the ∇-880 (insertion) allele. The -880Δ allele displayed increased gene expression by ~16.2% (on a C-399 background, p<0.001), as well as higher plasma NPY concentration by 39.4% (Δ/Δ, compared with ∇/Δ or ∇/∇, p=0.007).

Supplementary Table 1: Systematic polymorphism determination at the *NPY* locus in n=80 human subjects (2n=160 chromosomes). Characteristics of re-sequenced subjects in the study. Values are shown as mean ± SEM.

Variables	White (n=24)	Black (n=24)	Hispanic (n=16)	Asian (n=16)	Global (n=80)
Gender (M/F)	16/8	20/4	13/3	8/8	57/23
Age (years)	48±2.2	53±2.6	52±2.7	28±2.5	46±1.6
Hypertension (N/Y)	16/8	5/19	11/5	14/2	46/34
Height (m)	1.72±0.02	1.76±0.02	1.70±0.02	1.66±0.02	1.72±0.01
Weight (kg)	82±3.9	89±2.8	84±5.4	65±3.3	80±2.1
Body surface area (m^2)	1.95±0.06	2.08±0.04	1.99±0.08	1.73±0.05	1.94±0.03
Body mass index (kg/m^2)	28.8±1.7	28.7±1.2	28.7±1.3	23.4±0.9	27.5±0.8
Systolic BP (mmHg)	129±4.8	147±25.4	137±5.9	121±4.6	134±2.8
Diastolic BP (mmHg)	74±3.2	84±3.2	75±3.5	68±2.9	76±1.7

Supplementary Table 2: PCR and sequencing primers for polymorphism determination by re-sequencing the *NPY* gene. Primers are shown as 5' to 3'.

NPY P1	Sense	TGAAAGGGATGTTGGTCTCC
	Anti-sense	CTGAAAAAGAGCCACGCTTC
NPY P2	Sense	TTAACCACCGTCACTTTGGA
	Anti-sense	GACAACACCAAAGCCCAAGTA
NPY E1	Sense	GGCAGGAGACAAGAATCGTC
	Anti-sense	CATAGTCCGCTGGGGAGTG
NPY E2	Sense	CACTCCTGGGTTCTCTCTGC
	Anti-sense	ATCTGGGGATCACGACTCTG
NPY E3	Sense	GAAGGCAGGAATTTGACTAGGA
	Anti-sense	CCTCTGCCTGCTTCTTCATC
NPY E4	Sense	CCTGTCAACATCGTGGAATG
	Anti-sense	ATTAGCGAAACGAACCCTGA

Supplementary Table 3: PCR primers for SNP genotyping by SNaPshot (ABI). Primers are shown as 5' to 3'. The two promoter polymorphisms are amplified in one amplicon, while the other 3 SNPs are in the second amplicon.

Amplicon #	**SNPs**	**Primer**	**Sequence**
1	rs3037354,	Sense	CAGAACCCACATTCTCAACG
	rs16147	Anti-sense	TCCCTGCTTTCCTTCTTTCC
2	rs16140,	Sense	CACTCCTGGGTTCTCTCTGC
	rs16139, rs5573	Anti-sense	ATCTGGGGATCACGACTCTG

Supplementary Table 4: Primer information for genotyping.

A: SNPs information for SNaPshot (ABI) reaction. Primers are shown as 5’ to 3’. The two promoter polymorphisms are amplified in one amplicon, while the other 3 SNPs are in the second amplicon.

Position -cap site	RefSNP#	Major	Minor	Location	Codon-1	Codon-2	Amino acid change	Global MAF (n=80)	Flanking sequence	Variants	Read	Orientation	SNaPshot primer	Size
-880	rs3037354	TG	--	Promoter				32.5%	TTAGTAAATAAGACAGAAAC[TG/--] TTGCTCATTTAACCACCGTC	G/C	G/C	R	gacggtggttaaatgagcaa	20
-399	rs16147	T	C	Promoter				51.3%	CTACTCCGGCACCCAGTGGG[T/C] TGGTAGTCCTGTTGGCAGGA	C/T or A/G	C/T	F	Caggtgcttcctactccggca cccagtggg	30
1000	rs16140	C	G	Intron-1				31.2%	CAGAGGAGGGAGGTGCTGCG[C/G] GTGGGTGCTCTGAATCCCCA	G/C	G/C	F	Gaggaggagcgcgggggg cagaggagggaggtgctgcg	38
1071	rs16139	T	C	Exon-2	CTG	CCG	Leu/Pro	1.9%	ATGCTAGGTAACAAGCGAC[T/C] GGGGCTGTCCGGACTGACCC	C/T or A/G	C/T	F	Cgtccgttgagccttctgtgcct gcagATGCTAGGTAA CAAGCGAC	46
1201	rs5573	G	A	Exon-2	TCG	TCA	Ser/Ser	50.0%	GACATGGCCAGATACTACTC[G/A] GCGCTGCGACACTACATCAA	A/G or C/T	A/G	F	GCCGGACAACCCGG GCGAGGACGCACCA GCGGAGGACATGGC CAGATACTACTC	54

B: Primers for the genotyping of rs5574 by pyrosequencing.

Primer	ID	Sequence	Bp	Tm, °C	%GC
PCR F	F1	GGTTTTTATGCCTATTCCAAACTT	24	67.7	33.3
PCR R-5’-biotin	R1	TTCTGGGAACATTTTCTGTGC	21	69	42.9
Sequencing	S1	CCAGATATGGAAAACGAT	18	52.5	38.9

Supplementary Table 5: Characteristics of hypertensive (HT) and normotensive (NT) individuals in the blood pressure extremes.

Descriptive statistics		N	Mean ± SEM	*P*-value
Sex (M, F)	NT	553	M:230 F:323	0.02
	HT	574	M:201 F:373	
Height (inch)	NT	553	66.4±0.16	0.4
	HT	572	66.2±0.16	
Weight (lb)	NT	552	157.2±1.3	<0.001
	HT	573	190.7±2.0	
BMI (kg/m^2)	NT	552	25.0±0.2	<0.001
	HT	572	30.5±0.3	
Medications for HT (%)	NT	553	None	<0.001
	HT	574	37.1%	
SBP (mmHg)	NT	553	108.0±0.6	<0.001
	HT	574	146.3±0.6	
DBP (mmHg)	NT	553	60.1±0.1	<0.001
	HT	574	92.9±0.2	
Serum creatinine (mg/dl)	NT	543	0.96±0.009	0.38

	HT	574	0.95±0.008	
Serum glucose (mg/dl)	NT	542	87.6±0.7	<0.001
	HT	571	94.1±1.1	
Serum cholesterol (mg/dl)	NT	541	205.4±1.6	<0.001
	HT	574	225.1±1.8	
Serum HDL cholesterol (mg/dl)	NT	468	59.5±0.8	0.01
	HT	535	56.4±0.8	

Supplementary Table 6: Oligonucleotides for Electrophoretic Mobility Shift Assays (EMSA). They are shown as 5' to 3'. Polymorphisms are shown in **CAPITALIZED BOLD** type.

Oligonucleotide	Sequence
NPY880_Inser_EMSA_F:	ataagacagaaac**TG**ttgctcatttaac
NPY880_Inser_EMSA_R:	gttaaatgagcaa**CA**gtttctgtcttat
NPY880_Del_EMSA_F:	Ataagacagaaac__ttgctcatttaac
NPY880_Del_EMSA_R:	Gttaaatgagcaa__gtttctgtcttat
NPY C-399_EMSA_F:	tccggcacccagtggg**C**tggtagtcctgttggc
NPY C-399_EMSA_R:	gccaacaggactacca**G**cccactgggtgccgga
NPY 399T_EMSA_F:	tccggcacccagtggg**T**tggtagtcctgttggc
NPY 399T_EMSA_R:	gccaacaggactacca**A**cccactgggtgccgga

Supplementary Table 7: Summary of *NPY* SNP determination. The location and minor allele frequency for each polymorphism is given by population, and their positions are numbered upstream (-) or downstream (+) of the cap (transcription initiation) site. For each SNP, the reference number (RefSNP) is given where available in the public database. Nucleotide deletion is indicated by "–". In flanking sequence, SNPs are bracketed, and shown as major allele in UPPER CASE/minor in lower case. Exonic (transcribed) sequences are also shown as UPPER case.

#	Position-cap site		Allele		Location	Codon -1	Codon -2	Amino acid change	White (n=24)	Black (n=24)	Hispanic (n=16)	Asian (n=16)	Global (n=80)	Flanking sequence
			Major	Minor										
1	-880	rs3037354	TG (∇)	-- (Δ)	Promoter				29.2%	35.4%	28.1%	28.1%	31.9%	Ttagtaaataagacagaaac[TG/--]ttgctcatttaaccaccgtc
2	-602	rs17149106	G	T	Promoter				6.3%	2.1%	3.1%	3.1%	3.8%	Gaaaccacgggcgggggtgg[G/t]gtggggagcgcagctttggg
3	-560	New	G	A	Promoter				0.0%	2.1%	0.0%	0.0%	0.6%	Ccctctagccggagacttcc[G/a]gcagctgcctccgacttgtt
4	-464	rs36227310	G	C	Promoter				2.1%	10.4%	3.1%	0.0%	4.4%	Gtgcccggcgaggatgccgc[G/c]ctagtcgtggagatgcccca
5	-399	rs16147	C	T	Promoter				50.0%	58.3%	40.6%	37.5%	48.1%	Ctactccggcacccagtggg[C/t]tggtagtcctgttggcagga
6	1000	rs16140	C	G	Intron-1				35.4%	33.3%	25.0%	28.1%	31.3%	Cagaggagggaggtgctgcg[C/g]gtgggtgctctgaatcccca
7	1071	rs16139	T	C	Exon-2	CTG	CCG	Leu7Pro	2.1%	0.0%	3.1%	3.1%	1.9%	ATGCTAGGTAACAAGCGAC[T/c]GGGGCTGTCCGGACTGACCC
8	1135	rs5572	G	A	Exon-2	GCG	GCA	Ala28Ala	0.0%	12.5%	3.1%	0.0%	4.4%	CTGGGTGCGCTGGCCGAGGC[G/aTACCCCTCCAAGCCGGACAA
9	1201	rs5573	G	A	Exon-2	TCG	TCA	Ser50Ser	45.8%	37.5%	56.3%	68.8%	50.0%	GACATGGCCAGATACTACTC[G/a]GCGCTGCGACACTACATCAA
10	5298	rs35313836	10T	11T	Intron-2				10.4%	6.3%	12.5%	3.1%	8.1%	Ctttaaaagacttttttttt[T/-]ccagATATGGAAAACGATCc
11	5327	rs5574	C	T	Exon-3	TCC	TCT	Ser68Ser	43.8%	35.4%	43.8%	34.4%	39.4%	tccagATATGGAAAACGATC[C/t]AGCCCAGAGACACTGATTTC
12	7493	New	T	C	Exon-4	ATG	ACG	Met95Thr	2.1%	0.0%	0.0%	0.0%	0.6%	cagGCTTGAAGACCCTGCAA[T/c]GTGGTGATGGGAAATGAGAC
13	7545	rs5576	A	G	Exon-4/3'-UTR				0.0%	2.1%	0.0%	0.0%	0.6%	CCTTTTCCTATTTTCAGCCC[A/g]TATTTCATCGTGTAAAACGA
14	7597	New	A	G	Exon-4/3'-UTR				0.0%	2.1%	0.0%	0.0%	0.6%	TCCTACCAATGCATGCAGCC[A/g]CTGTGCTGAATTCTGCAATG
15	7680	rs16475	A	G	Beyond 3'-End				10.4%	0.0%	9.4%	0.0%	5.0%	TATCATGCATTCaaaagtgt[A/g]tcctcctcaatgaaaaatct
16	7711	rs16126	T	C	Beyond 3'-End				2.1%	16.7%	0.0%	0.0%	5.6%	Tgaaaaatctattacaatag[T/c]gaggattattttcgttaaac

Supplementary Table 8: Six *NPY* SNPs genotyped in twin and sibling pairs, and population blood pressure extremes. These subjects were self-identified as being of European ancestry. Base position is numbered upstream (-) or downstream (+) of the cap site. NCBI RefSNP is the reference number in NCBI SNP database. MAF: Minor allele frequency. HWE: Hardy-Weinberg Equilibrium. MAF and HWE data came from the resequence of 24 white individuals.

Location	**Position**	**Allele**		**Amino acid**	**NCBI**	**MAF**	**HWE**	
		Major	**Minor**	**change**	**RefSNP**	**(%)**	χ^2	***P*-value**
Promoter	-880	TG (∇)	-- (Δ)	-	rs3037354	29.2	0.375	0.829
Promoter	-399	C	T	-	rs16147	50	0.043	0.978
Intron-1	1000	C	G	-	rs16140	35.4	0.518	0.772
Exon-2	1071	T	C	Leu7Pro	rs16139	2.1	0.011	0.995
Exon-2	1201	G	A	Ser50Ser	rs5573	45.8	0.734	0.693
Exon-3	5327	C	T	Ser68Ser	rs5574	43.80	0.734	0.693

Supplementary Table 9: *NPY* polymorphism distributions and Hardy-Weinberg equilibrium in sibs and twins.

	rs3037354	**rs16147**	**rs16140**	**rs16139**	**rs5573**	**rs5574**
Major/Major	194	106	174	390	105	110
Major/Minor	183	209	201	29	210	218
Minor/Minor	40	103	44	0	104	91
Chi-square	0.111	0.000	1.591	0.037	0.002	0.765
P value	0.946	1.000	0.451	0.982	0.999	0.682

Supplementary Table 10: *NPY* haplotype distribution in sibs and twins. The 5 variants used in constructing these haplotypes (by the HAP algorithm) were (5'→3'): rs3037354 (∇-880Δ, TG/--), rs16147 (C-399T), rs16140 (A1000G), rs16139 (T1071C, signal peptide Leu7Pro), rs5573 (G1201A), and rs5574 (C5327T).

Haplotypes	**2n (chromosomes)**	**% of chromosomes**
∇CCTAT	362	43.2
ΔTGTGC	251	29.95
∇TCTGC	114	13.6
∇CCTAC	28	3.34
∇TCCGC	25	2.98
ΔCGTAT	23	2.74
ΔTCTGC	10	1.19
∇CGTAC	5	0.6
∇TCTGT	5	0.6
∇CGTGT	2	0.24

∇TCMGT	2	0.24
∇TGTGC	2	0.24
∇TGTGT	2	0.24
ΔTGTGT	2	0.24
∇CCTGT	1	0.12
∇CCMGC	1	0.12
ΔCGTGC	1	0.12
∇TCMAT	1	0.12
∇TGTAC	1	0.12

Supplementary Table 11. *NPY* polymorphism HWE and distributions in white blood pressure extremes. Chi-square and p values are shown for HWE.

SNP	rs3037354	rs16147	rs16140	rs16139	rs5573	rs5574
Major/Major	622	310	586	1057	313	288
Major/Minor	412	562	435	62	551	556
Minor/Minor	84	249	100	1	256	267
Chi-square	1.867	0.036	2.197	0.009	0.205	0.002
P value	0.393	0.982	0.333	0.996	0.903	0.999

Supplementary Table 12 *NPY* haplotype distributions in white blood pressure extremes. The 5 variants used in constructing these haplotypes (by the HAP algorithm) were (5’→3’): rs3037354 (-880 TG/--, ∇/Δ), rs16147 (-399C/T), rs16140 (A1000G), rs16139 (T1071C, signal peptide Leu7Pro), rs5573 (G1201A), and rs5574 (C5327T).

Haplotypes	2n (chromosomes)	% of chromosomes
∇CCTAT	1033	46.1
Δ TGTGC	542	24.2
∇TCTGC	380	16.9
∇CCTAC	91	4.1
∇TCCGC	54	2.4
∇CGTAT	37	1.7
∇TGTGC	27	1.2
ΔTCTGC	19	0.8
Δ TGTGT	13	0.6
∇TCTGT	8	0.4
∇CCTGC	6	0.3
∇CGTGT	4	0.2
∇TCTAC	4	0.2
∇TCCGT	4	0.2
∇CGTGC	3	0.1
∇CGCAT	2	0.1
∇TGTAC	2	0.1
ΔTGTAC	2	0.1

∇CCTGT	1	0.0
∇CCCAT	1	0.0
∇CCCGC	1	0.0
∇CGTAC	1	0.0
∇CGCGC	1	0.0
∇TCTAT	1	0.0
∇TGCGC	1	0.0
∆CCTAC	1	0.0
∆CCTGC	1	0.0
∆TCTAC	1	0.0
∆TCTAT	1	0.0

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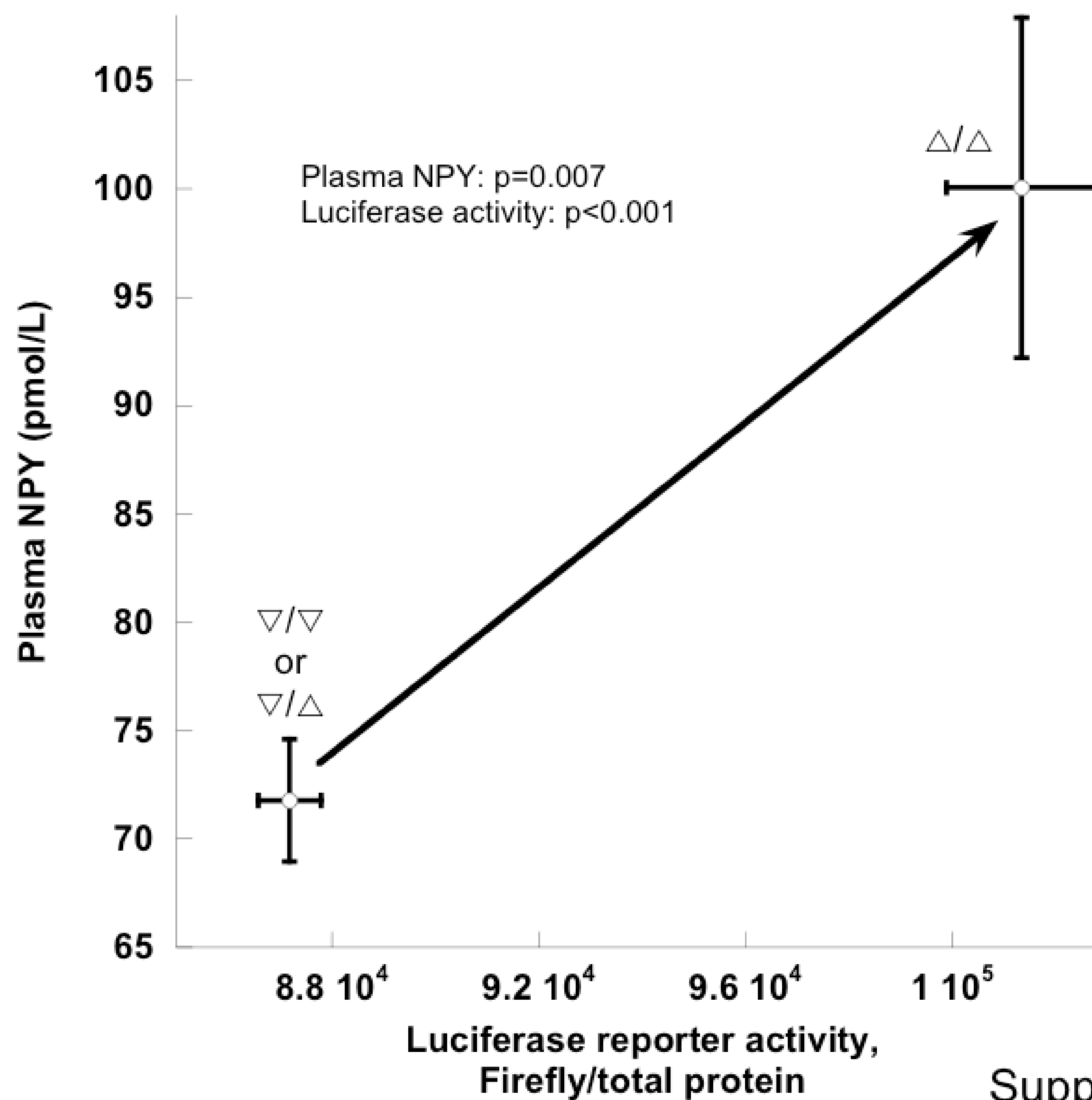
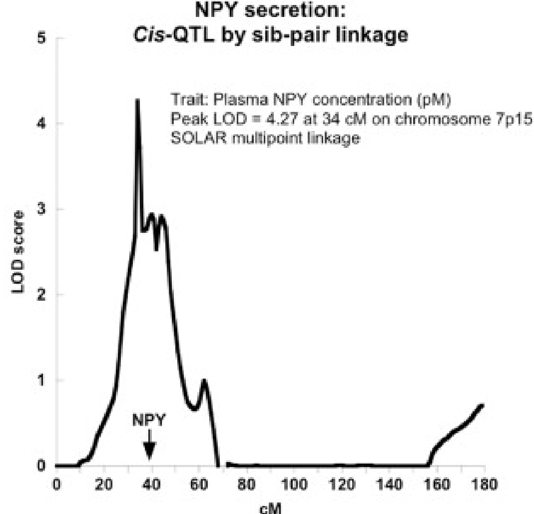
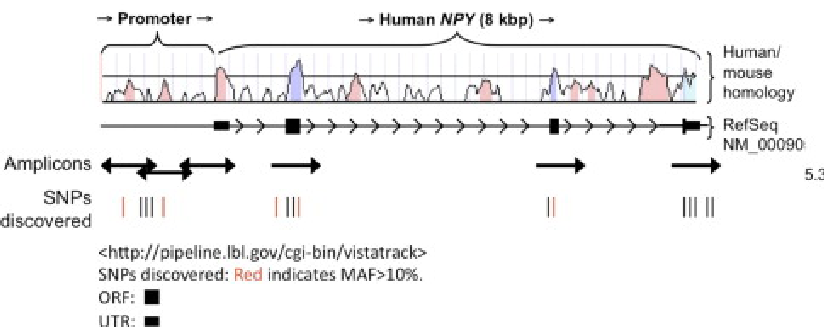
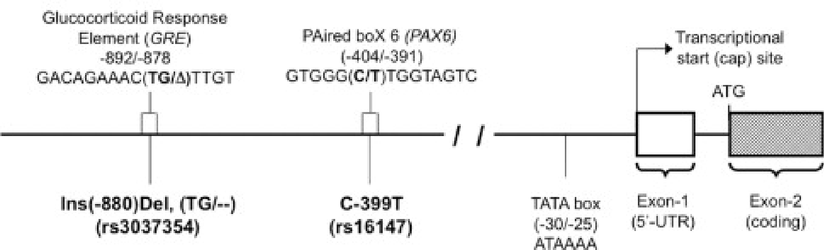
***NPY* promoter variation:**
Coordinate* directional functions *in cella* and *in vivo

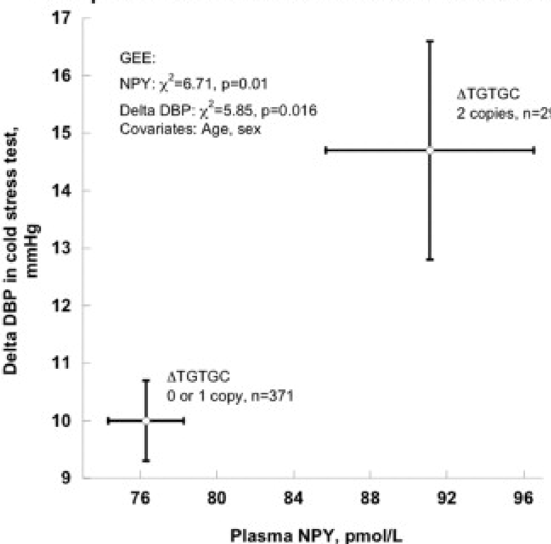


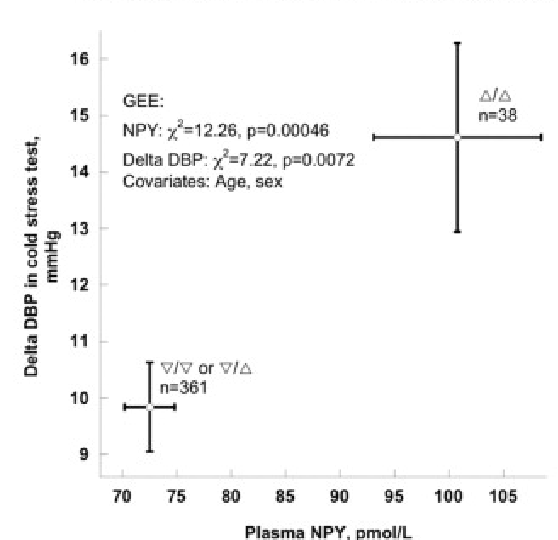
Supplementary Figure 6

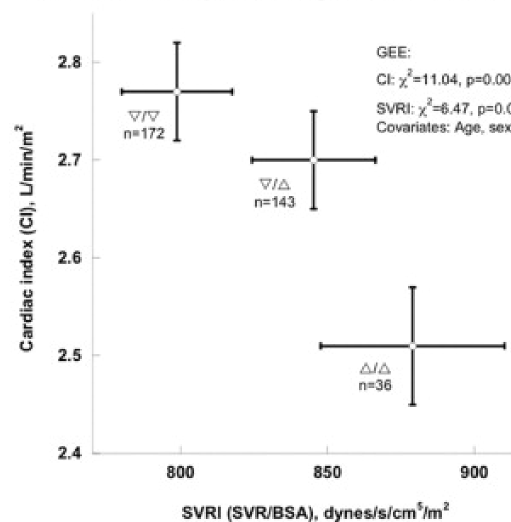

A
NPY secretion:
Cis-QTL by sib-pair linkage
Trait: Plasma NPY concentration (pM)
Peak LOD = 4.27 at 34 cM on chromosome 7p15
SOLAR multipoint linkage
LOD score
NPY
cM
0
1
2
3
4
5
0
20
40
60
80
100
120
140
160
180


B
Human *NPY*: Systematic polymorphism determination by re-sequencing
→ Promoter →
→ Human *NPY* (8 kbp) →
Human/ mouse homology
RefSeq NM_00090
5.3
Amplicons
SNPs discovered
<http://pipeline.lbl.gov/cgi-bin/vistatrack>
SNPs discovered: Red indicates MAF>10%.
ORF:
UTR:


C
Genetic variation in the proximal human *NPY* promoter: Impact on domains and motifs
Glucocorticoid Response Element (*GRE*)
-892/-878
GACAGAAAC(**TG/Δ**)TTGT
PAired boX 6 (*PAX6*)
(-404/-391)
GTGGG(**C/T**)TGGTAGTC
Transcriptional start (cap) site
ATG
Ins(-880)Del, (TG/--)
(rs3037354)
C-399T
(rs16147)
TATA box
(-30/-25)
ATAAAA
Exon-1
(5'-UTR)
Exon-2
(coding)
NCBI RefSeq clone: NM_000905.2
Bold: Polymorphic base(s).
G/C-rich domains: -49/-41,-59/-54,-105/-98,-119/-111,-126/-121, -188/-181, -213/-206, -251/-246, -335/-329, -482/-475, -494/-489, -518/-513, -615/-606


A
Association of NPY haplotype ΔTGTGC
with plasma NPY and delta DBP in cold stress test
GEE:
NPY: χ2=6.71, p=0.01
Delta DBP: χ2=5.85, p=0.016
Covariates: Age, sex
ΔTGTGC
2 copies, n=29
ΔTGTGC
0 or 1 copy, n=371
Delta DBP in cold stress test, mmHg
Plasma NPY, pmol/L
17
16
15
14
13
12
11
10
9
76
80
84
88
92
96


B
Association of NPY promoter polymorphism ▽-880△
with plasma NPY and ΔDBP in cold stress test
GEE:
NPY: χ2=12.26, p=0.00046
Delta DBP: χ2=7.22, p=0.0072
Covariates: Age, sex
△/△
n=38
▽/▽ or ▽/△
n=361
Delta DBP in cold stress test, mmHg
Plasma NPY, pmol/L
16
15
14
13
12
11
10
9
70
75
80
85
90
95
100
105


C
NPY promoter variant ▽-880△ in twin and sibling pairs:
Deletion variant predicts higher SVRI with lower CI
GEE:
CI: χ2=11.04, p=0.004
SVRI: χ2=6.47, p=0.039
Covariates: Age, sex
▽/▽
n=172
▽/△
n=143
△/△
n=36
Cardiac index (CI), L/min/m2
SVRI (SVR/BSA), dynes/s/cm5/m2
2.8
2.7
2.6
2.5
2.4
800
850
900

A

NPY promoter common variant ∇-880Δ (Ins/Del, TG/--): Disruption of a glucocorticoid response element (GRE) motif

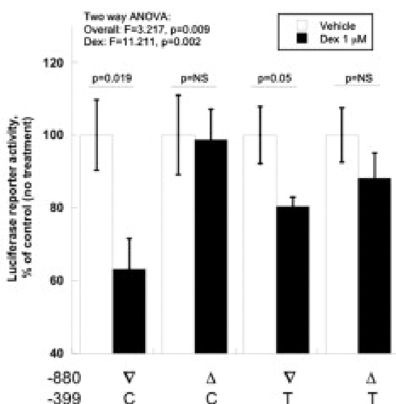
***GRE* and ∇-880Δ across species**

	6-bp direct / 3-bp spacer / 6-bp inverted	Match
GRE motif	GGTACANNNTGTTCT	Match
Human TG	**G**AC**A**G**A**AAC**TGTT**G**T**	8/12
Human --	**G**AC**A**G**A**AAC--**TT**G**T**	6/12
Chimp	**G**AC**A**G**A**AAC**TGTT**GC	7/12
Orangutan	**G**AC**A**G**A**AAC--**TT**GC	5/12
Marmoset	**GG**G**A**G**A**AAT**TGTT**G**T**	9/12
Horse	CAG**A**G**A**AAT**TG**C**T**G**T**	6/12
Dog	AGG**A**G**A**AAT**T**CCCG**T**	4/12

∇-880Δ (rs3037354)

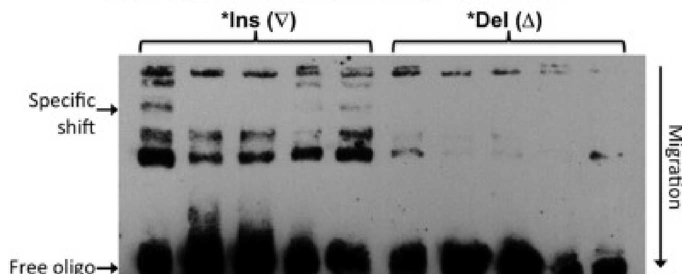
Bold: Sequence identity with GRE motif.
GRE: Glucocorticoid Response Element.

Glucocorticoid effect on *NPY* promoter ∇-880Δ/C-399T haplotype expression in chromaffin cells



B

Glucocorticoid receptor interaction with *NPY* promoter Ins/Del variant ∇-880Δ (TG/--): EMSA.



PC12 NX	+	+	+	+	+	+	+	+	+	+
Labeled (*)	I	I	I	I	I	D	D	D	D	D
Unlabeled	–	I	D	–	–	–	I	D	–	–
Anti-GR	–	–	–	+	–	–	–	–	+	–
Ctl antibody	–	–	–	–	+	–	–	–	–	+

I: Insertion of TG (∇)
D: Deletion of TG (Δ)
GR: Glucocorticoid Receptor
Ctl: Control
NX: Nuclear Extract

A

NPY haplotype ΔTGTGC on BP in the population

NPY haplotype ΔTGTGC on blood pressure in the population

ANOVA:
SBP: F=8.67, p=0.003
DBP: F=5.77, p=0.016
Covariates: Age, sex

ΔTGTGC present
n=475

ΔTGTGC absent
n=646

DBP, mmHg

SBP, mmHg

NPY haplotype ΔTGTGC on BP status in the population

NT
HT

Fisher's exact test: p=0.01

n=336
n=310
n=213
n=262

% of individuals by BP status (NT or HT) for *NPY* haplotype ΔTGTGC

Absent
Present

Haplotype ΔTGTGC

B

NPY promoter variant ∇-880Δ in the population: Deletion variant predicts higher SBP/DBP and hypertension

NPY promoter polymorphism ∇-880Δ (rs3037354) on blood pressure in BP extremes

ANOVA:
SBP: F=8.54, p=0.004
DBP: F=4.08, p=0.044

∇/Δ or Δ/Δ
n=496

∇/∇
n=622

DBP, mmHg

SBP, mmHg

NPY promoter polymorphism ∇-880Δ (rs3037354) on BP status in the population

NT
HT

Fisher's exact test: p=0.029

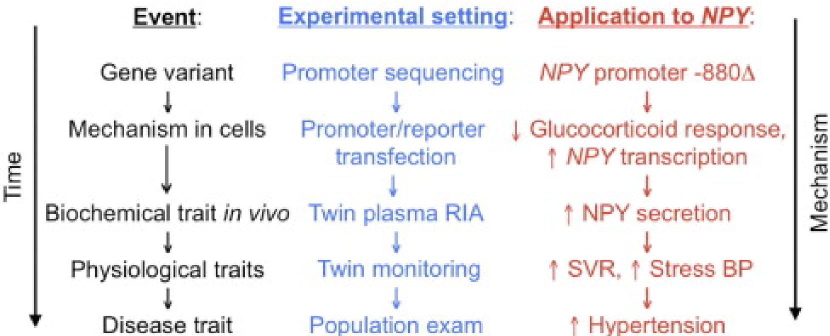
n=320
n=302
n=225
n=269

% of individuals by BP status (NT or HT) for each NPY promoter In/Del -882 genotype

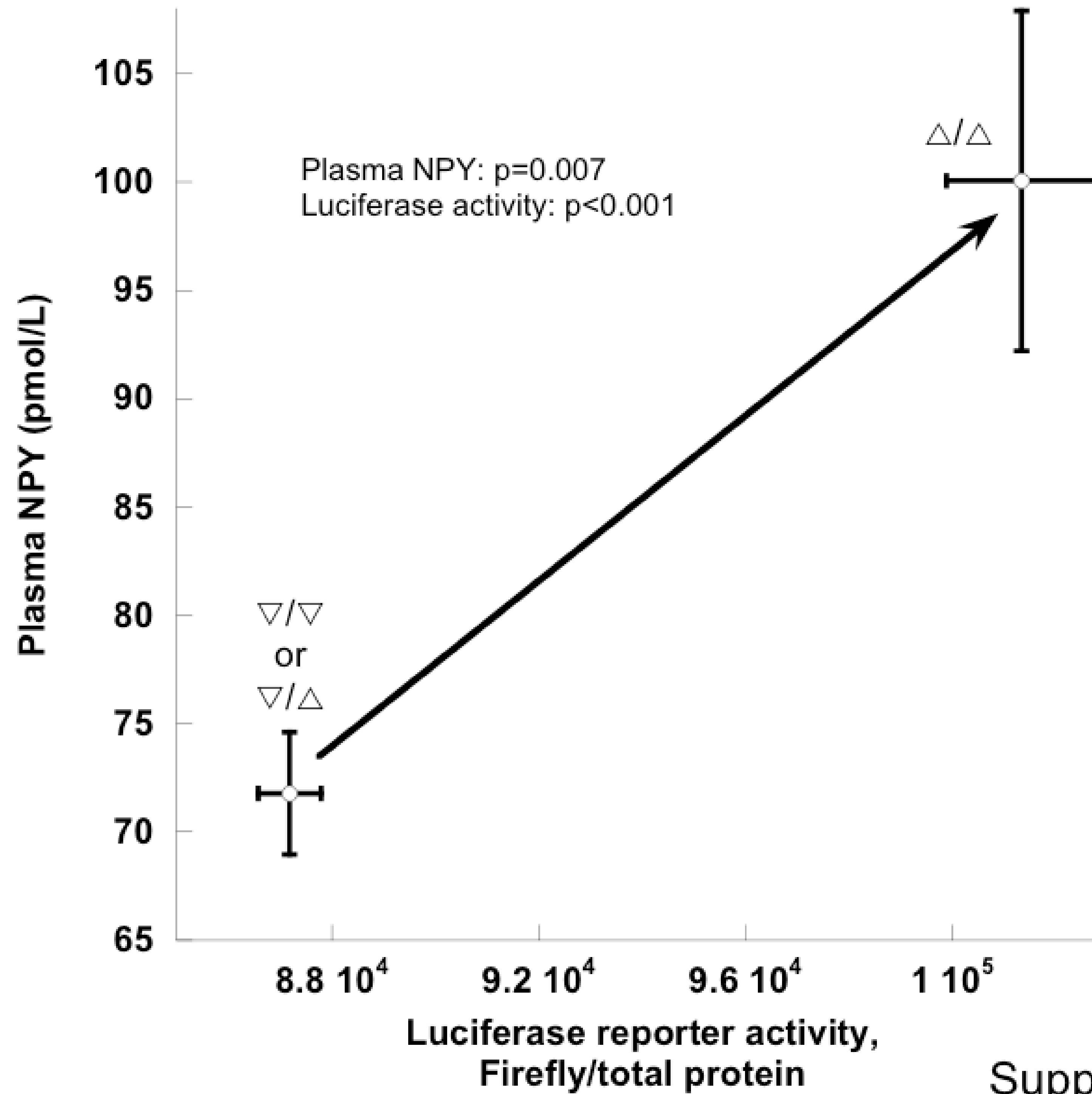
∇/∇
∇/Δ or Δ/Δ

Genotype

Human *NPY* promoter genetic variant ∇-880Δ:
Schema for effects on autonomic and disease traits



***NPY* promoter variation:**
Coordinate* directional functions *in cella* and *in vivo



Supplementary Figure 6