Published in final edited form as:
J Neurochem. 2013 December ; 127(6): . doi:10.1111/jnc.12346.

Genetic variation at the delta-sarcoglycan (*SGCD)* locus elevates heritable sympathetic nerve activity in human twin pairs

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Abstract

The Syrian Cardiomyopathic Hamster (BIO-14.6/53.58 strains) model of cardiac failure, resulting from naturally occurring deletion at the *SGCD* (delta-sarcoglycan) locus, displays widespread disturbances in catecholamine metabolism. Rare Mendelian myopathy disorders of human *SGCD* occur, though common naturally occurring *SGCD* genetic variation has not been evaluated for effects on human norepinephrine (NE) secretion. This study investigated the effect of *SGCD* genetic variation on control of NE secretion in healthy twin pairs. Genetic associations profiled SNPs across the *SGCD* locus. Trait heritability (h^2) and genetic covariance (pleiotropy; shared h^2) were evaluated. Sympathochromaffin exocytosis *in vivo* was probed in plasma by both catecholamines and CHGB. Plasma NE is substantially heritable (P=3.19E-16, at 65.2±5.0% of trait variance), sharing significant ($P<0.05$) genetic determination with circulating and urinary catecholamines, CHGB, eGFR and several cardio-metabolic traits. Participants with higher pNE showed significant ($P<0.05$) differences in several traits, including increased BP and hypertension risk factors. Peak *SGCD* variant rs1835919 predicted elevated systemic vascular compliance, without changes in specifically myocardial traits. We used a chimeric regulated secretory pathway photoprotein (CHGA-EAP) to evaluate the effect of SGCD on the exocytotic pathway in transfected PC12 cells; in transfected cells, expression of SGCD augmented CHGA trafficking into the exocytotic regulated secretory pathway. Thus our investigation determined human NE secretion to be a highly heritable trait, influenced by common genetic variation within the *SGCD* locus. Circulating NE aggregates with BP and hypertension risk factors. Additionally, coordinate NE and CHGB elevation by rs1835919 implicates exocytosis as the mechanism of release.

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CONFLICTS OF INTEREST DISCLOSURE.
The authors have no conflicts of interest to disclose.

Keywords

Norepinephrine; catecholamine; delta-sarcoglycan; secretion; cytoskeleton

INTRODUCTION

The sympathetic neurotransmitter norepinephrine (NE, noradrenaline) plays an essential role in circulatory homeostasis, with actions on both the heart and vessels (Parati & Esler 2012). Using plasma concentration as a rough index of sympathetic activity, investigators have shown NE secretion governs diverse metabolic and hemodynamic pathways.

In congestive heart failure, sympathetic indexes are systematically elevated as a function of severity, including steady-state plasma NE (pNE) concentration, with increased NE spillover from sympathetic terminals, especially cardiac and renal spillover (Hasking *et al.* 1986), as well as epinephrine release from the adrenal medulla, and finally decreased plasma clearance of the amines.

The Syrian Cardiomyopathic Hamster (SCH; BIO 14.6 and 53.58 strains) is a widely studied experimental model of hereditary hypertrophic cardiomyopathy progressing to heart failure and its associated autonomic dysfunction. These animals are characterized by substantially increased catecholamine secretion, turnover, and elevated plasma concentrations (Yamada *et al.* 1997) in the face of congestive heart failure. Such phenotypic traits of the SCH begin relatively early in life, from 40 days of age onward, then increasing until death or sacrifice. Literature on the SCH model is substantial.

Italian (Nigro *et al.* 1997) and Japanese (Okazaki *et al.* 1996) investigators demonstrated that the cardiomyopathic traits of the SCH emerge from a single deletion mutation across the promoter and exon-1 in the delta-sarcoglycan (*SGCD*) locus, effectively deleting expression of SGCD mRNA and protein from myocytes (Escobales & Crespo 2008, Nigro et al. 1997). Thus, the SCH constitutes a Mendelian model for heart failure and its associated disorders of catecholamine storage and release (Escobales & Crespo 2008, Nigro et al. 1997, Sole *et al.* 1975, Vainzof *et al.* 2008, Yamada et al. 1997). Targeted ablation of the *Sgcd* gene in the mouse results in cardiomyopathy (Coral-Vazquez *et al.* 1999), while inactivating mutations in human *SGCD*, on chromosome 5q33, have been associated with autosomal recessive limb-girdle muscular dystrophy or dilated cardiomyopathy <http://omim.org/entry/601411>.

Thus profound inactivating mutations of *SGCD* eventuate in cardiomyopathy and disordered sympathetic activity, in both animal models and humans. However, subtle naturally occurring alterations (i.e., common allelic variants) of *SGCD* have not been evaluated for effects upon NE secretion or cardiac function. Here we coupled autonomic phenotyping with extensive genotyping at the *SGCD* locus, to probe potential involvement of *SGCD* in control of NE secretion in a series of healthy twin pairs and siblings. We found that NE secretion is a heritable trait influenced by common genetic variation within *SGCD*.

MATERIALS AND METHODS

Twin and sibling subjects

The UCSD twin/sibling study has previously been described (Davis *et al.* 2012). Twin and sibling participants were recruited from southern California by access to a population birth record-based twin registry (Cockburn *et al.* 2002), as well as by newspaper advertisement, as described previously (Zhang *et al.* 2004). The protocol was approved by the UCSD Human Research Protection Program, and each subject gave written informed consent prior

to participation. Subjects included dizygotic (DZ) and monozygotic (MZ) twin pairs. Zygosity of twins was confirmed genetically by microsatellite and single nucleotide polymorphisms (SNP) markers (Zhang et al. 2004). Initially ethnicity was established by self-identification, including information on geographic origin of both parents and all four grandparents, and only individuals of Caucasian or Hispanic (Mexican-American) ancestry/ethnicity were included here. The age of the subjects ranged from 14 to 78 years. Phenotyping (biochemical and physiological) was conducted as previously described (Zhang et al. 2004). Blood pressure (BP) status (high vs. normal) was defined by history (medical record or self-report), presence of antihypertensive medications, and measurement of seated BP by arm cuff (hypertension: either/or ≥140/≥90 mmHg systolic BP (SBP)/diastolic BP (DBP), or both). None of the subjects had a history of cardiovascular disease or renal failure, and serum creatinine concentrations were ≤1.5 mg/dl.

Twin/sibling physiological phenotyping

Brachial cuff BPs with SBP/DBP measured as K1/K4, heart rate, cardiac output, stroke volume, systemic vascular resistance (SVR), and systemic vascular compliance (SVC) were obtained noninvasively in seated subjects in triplicate using an oscillometric device (DynaPulse; PulseMetric Inc., San Diego, CA) as described (Davis *et al.* 2012). Triplicate values (within ±10% of individual's mean) were averaged. We and others previously validated DynaPulse measurements against more invasive devices (Brinton *et al.* 1996, Davis et al. 2012).

Biochemical phenotyping

Subjects were instructed to fast during the 6 h preceding the evaluation. Fasting blood and urine samples were collected from each subject. Plasma and urine samples were quickly frozen to −70°C, in preparation for catecholamine concentration assessment. A sensitive radioenzymatic assay was used to measure plasma and urine catecholamines (dopamine (DA), NE, and epinephrine (EPI)) through the catechol-O-methylation process (Ziegler *et al.* 1988). The radioenzymatic assay for catecholamines involved transfer of a ^{3}H label to catecholamines from S-adenosylmethionine during O-methylation, mediated by the enzyme catechol-O-methyltransferase (COMT). Prior to O-methylation, plasma catecholamines were extracted into dilute acetic acid to remove COMT inhibitors in plasma. Assay sensitivities (lower limits of detection) were 10 pg for NE and 6 pg for EPI. Intra-assay coefficients of variation were 4% for NE and 13% for EPI, while inter-assay coefficients of variation were 10% for NE and 13% for EPI. Urine catecholamine values were normalized to the level of creatinine excretion of the subject's same sample. Plasma lipids were measured as previously described (Wessel *et al*. 2007). Plasma insulin, leptin, and renin were measured by immunoassay. Plasma glucose and creatinine were measured by autoanalyzer (Beckman-Coulter; Brea, CA).

Heritability(h^2)

Estimates of heritability ($h^2 = V_G/V_P$, where V_G is additive genetic variance and V_P is total phenotypic variance) were obtained with variance-component methods implemented in Sequential Oligogenic Linkage Analysis Routines (SOLAR) (Almasy & Blangero 1998), available at <http://solar.txbiomedgenetics.org>. This method maximizes the likelihood assuming a normal distribution of phenotypes in twin pairs (MZ versus DZ). The null hypothesis (H_0 of $h^2=0$) is tested by comparing the full model, which assumes non-zero genetic variation (V_G), and a reduced model, which assumes no genetic variation, by a likelihood ratio test. SOLAR was also used to evaluate whether allelic variation at the locus contributed to a significant fraction of the trait heritability (i.e., locus-specific V_G) by

comparing models including or excluding the genotype as a covariate. Heritability estimates were adjusted for age and sex, since these covariates affected several traits.

Pleiotropy

Pleiotropy (genetic covariance for two correlated, heritable traits) was estimated as the parameter RhoG (ρ_G) in SOLAR, as described (Wessel *et al.* 2007). SOLAR was also used to estimate the environmental covariance, as parameter RhoE (ρ_E).

Genotyping

Genomic DNA was isolated from blood leukocytes on Qiagen columns, after proteinase K digestion of protein, as previously described (Zhang *et al.* 2004). SNP genotyping across the *SGCD* locus was accomplished using the Illumina 610-Quad genotyping array. Results across the locus, centered on the peak association at rs1835919, were displayed by local SNAP (SNP Annotation and Proxy Search) plot as described at <http://www.broadinstitute.org/mpg/snap/ldplot.php>. Marker-on-trait significance was estimated using the variance components software MERLIN <http://www.sph.umich.edu/csg/abecasis/Merlin/>, which accounts for intra-twin-pair correlations. Analyses were adjusted by age and sex. Marker-on-trait significance was also estimated by GEE (generalized estimating equations).

Calculations

Body surface area (BSA, in m^2), as a measure of overall body size, was estimated by the Mosteller formula <http://www.halls.md/body-surface-area/refs.htm>. Glomerular filtration rate (GFR) was estimated from ethnicity, sex, and plasma creatinine using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) algorithm (Levey *et al.* 2009). Endogenous insulin sensitivity (or resistance) was estimated from fasting plasma glucose and insulin values by QUICKI (QUantitative Insulin sensitivity ChecK Index; an index of insulin sensitivity), calculated as previously described (Lee *et al.* 2004).

Statistical analyses

Descriptive and inferential statistics (mean ± SEM) were computed by GEE using IBM SPSS predictive analytics software, to account for intra-twin-pair correlations. GEE also tested for allele and diploid genotype effects on traits. The SNP was defined as a 3-level variable representing the 3 possible genotypes (homozygous variant, heterozygous, and homozygous wild-type), initially assuming an additive effect in the model. Analyses were adjusted for age and sex. False Discovery Rate (FDR): When testing the effects of a gene on multiple correlated traits, we estimated the FDR, in order to minimize false negative results while maximizing true positive results, using the Excel calculator of FDR from a distribution of p-values, at <http://www.rowett.ac.uk/~gwh/fdr.html>.

Bioinformatics

We viewed the likely exon/intron structure for human *SGCD*, as well as putative promoter domains, at <http://genome.ucsc.edu>, focusing on long (~887 kbp) genomic clone uc0031wa.1 (in which the peak trait-associated region is in intron-4), or shorter RefSeq clone NM_000337 (~447 kbp), in which the peak trait-associated region is in the distal promoter, ~200 kbp upstream of exon-1. Transcription factor binding or epigenetic marks, documented by Chip-Seq in a variety of cell lines, were explored on the EnCode displays at <http://genome.ucsc.edu/ENCODE>. Transcription factor motif matches in the region were evaluated further on CONSITE (Sandelin *et al.* 2004) at <http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite>. Chromatin Conformation Capture (“Hi-C”) <http://chromosome.sdsc.edu/mouse/hi-c/index.html>, evaluated the boundaries of regions subject

to local *trans*-interactions, based on interactions in human embryonic stem cells and human IMR90 fetal lung fibroblasts. Evidence for *cis*-QTLs (sometimes known as allelic expression imbalance) was generated on the eQTL browser at the Pritchard lab from the University of Chicago, linking transcriptome and GWAS human studies <http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/>.

Construction of expression plasmids

The pCMV (pcDNA6 vector) eukaryotic expression plasmid for a chromogranin A (CHGA) / secretory embryonic alkaline phosphatase (CHGA-EAP) chimera was prepared as previously described (Taupenot 2007, Taupenot *et al.* 2002). Plasmid constructs were verified by restriction digestion and nucleotide sequence analysis. The full-length human SGCD cDNA (SC309561) was obtained in a pCMV (pCMV6-XL vector) eukaryotic expression plasmid, from Origene <www.origene.com>.

Cell culture and transient transfections

PC12 cells were cultured in Ham's F12K medium supplemented with 15% heat-inactivated horse serum and 2.5% heat-inactivated fetal bovine serum (Gemini Bioproducts), streptomycin (100 g/ml), and penicillin (100 units/ml) (Invitrogen). Supercoiled plasmid DNA for transfection was grown in Escherichia coli strain DH5α (Invitrogen) and purified on columns (Qiagen). Two days before transfection, PC12 cells were split onto either poly-L-lysine (Sigma) plus collagen (Upstate)-coated 18-mm round glass #1.5 coverslips (Fisher) in 12-well Costar plates or onto poly-L-lysine-coated 6- or 12-well Costar plates. Cells were co-transfected with pCMV-CHGA-EAP and pCMV-SGCD at 1.25 μg (12-well plate) or 2 μg (6-well plate) of supercoiled plasmid DNA per well, using a high efficiency cationic scaffold method (GenePorter II, Gene Therapy Systems). Five hours after beginning the transfection, culture medium was replaced and cells were further cultured for 22 or 48 h.

Perturbation of secretory protein trafficking by SGCD: Chemiluminescent detection of secretory protein/embryonic alkaline phosphatase (EAP) chimera secretion by PC12 cells

The protocol describing the chemiluminescent assay used to detect secretagogue-stimulated secretion of CHGA-EAP from PC12 cells, co-transfected with pCMV-SGCD and pCMV-CHGA-EAP, was previously published (Taupenot 2007). Briefly, detection of EAP enzymatic activity release from CHGA–EAP chimera-expressing PC12 cells was achieved using the chemiluminescent substrate CSPD (Phospha-Light, Applied Biosystems, Foster City, California) in a Luminometer Autolumat 953 (EG&G Berthold). EAP activity was measured in the culture supernatant and cell lysate. Exocytotic secretion of the transfected/expressed EAP chimeras was provoked by the potent regulated pathway stimulus Ba^{2+} (2 mmol/L), which acts by blocking efflux of cellular K^+ and hence cell membrane repolarization. The secretion rate was calculated from supernatant EAP activity as a percentage of total enzymatic activity (cell plus supernatant). The “sorting index” was calculated as a function of increase in secretion rate after stimulation of the regulated exocytotic pathway by Ba^{2+}: [stimulated minus basal]/basal.

RESULTS

Role of heredity and pleiotropy in norepinephrine secretion

Table S1 presents heritability (h^2) for each trait estimated from twin and sibling pair variance components, expressed as a % of trait variation (h^2 scaled from 0 to 100%). Circulating NE heritability is also exhibited graphically, along with several cardio-metabolic traits, in Figure 1a. Heritability of NE secretion was substantial (P=3.19E-16), at 65.2±5.0% of trait variance. Heritability was also substantial for cardio-metabolic traits listed in Table

S1, including BP and cardiovascular risk factors, as well as physical (i.e., body mass index (BMI)) and a number of biochemical (e.g., catecholamine) traits.

Correlations (Pearson) of NE secretion with other specific traits are also shown in Table S1. Hemodynamic/autonomic and biochemical traits displayed several correlating phenotypes with pNE. Correlations of significance were found for a variety of traits, including SBP and DBP, vascular compliance and resistance, baroreceptor slope (during both upward and downward BP deflections), circulating and urinary catecholamines, $CHGB_{312-331}$, active renin, free fatty acids (pFFA), and estimated GFR (eGFR, by CKD-EPI algorithm).

Heritability varied among traits correlating with circulating NE (Table S1); we then examined these correlated traits for shared genetic determination (pleiotropy, RhoG) with pNE (Table S1, Figure 1b). Results indicate that pNE shares significant genetic determination (RhoG) with BMI, SVC, SVR, circulating and urinary catecholamines, $CHGB_{312-331}$, eGFR, and plasma active renin. By contrast, several traits shared significant environmental determination (environmental codetermination, RhoE) with pNE: DBP, circulating and urinary catecholamines, pFFA, and eGFR. Circulating and urinary catecholamines and eGFR traits displayed both shared hereditary and environmental co-determination with pNE.

Figure 1b displays the shared hereditary co-determination, without significant environmental co-determination, for circulating NE with plasma EPI and $CHGB_{312-331}$. Environmental co-determination, without significant shared hereditary co-determination, was noted for eGFR (by CKD-EPI algorithm), plasma active renin, and SVC, as displayed in Figure 1b.

Trait aggregation with norepinephrine secretion: Norepinephrine quantiles

Table S2 provides a description of the twin/sibling cohort, divided into 2 groups (quantiles, upper and lower) stratified around the pNE median. Plasma NE increased with age and was higher in men than women. Participants with higher pNE showed significant ($P<0.05$) differences across several traits, including increased BP levels and hypertension risk factors: baroreceptor slope, catecholamines, pulse pressure, plasma insulin, pFFA, total cholesterol, triglycerides, plasma neuropeptide Y (pNPY), and eGFR (by CKD-EPI algorithm). However, subjects with higher pNE values did not show evidence of intrinsic cardiac pathology. Several cardiac traits, including cardiac output, cardiac index, stroke volume, and stroke volume index, were not affected by pNE level increases.

Evidence showing significant aggregation of other secreted catecholamines (EPI and DA) with increased circulation of NE is found in Figure 2a. Correspondingly, Figure 2b displays pNE effects on BP of hypertensive versus normotensive subjects, with significant elevation in hypertension (P=2.00E-03). Figure 2c further shows the effects of pNE distribution for the two major BP characteristics: SBP and DBP. Figure 2d illustrates that pNE and pNPY increase coordinately. Baroreceptor slope (upward and downward deflections) aggregated with pNE (Figure 2e), such that elevated NE secretion was found in subjects with lower/impaired baroreflex function. Metabolic traits, pFFA and total cholesterol, also rose with pNE level (Figure 2f). Figure 2g demonstrates the direct correspondence of pNE with urinary excreted NE, though an inverse effect of pNE level on eGFR (by CKD-EPI algorithm).

Human *SGCD* genomic region and norepinephrine secretion

Genetic markers were typed across the local *SGCD* region on chromosome 5q33; a SNAP plot, displaying local significance for genetic determination of NE secretion, is displayed in Figure 3a. The ~100 kbp peak association occurred for SNP rs1835919 in the *SGCD* region,

located in intron-4 of long genomic clone uc0031wa.1; alternatively, the same SNP peak is found in the distal promoter of RefSeq clone NM_000337, ~200 kbp upstream of exon-1.

The minor (G) allele frequency of the peak trait-associated variant (rs1835919) was 19%, and the 3 diploid genotypes (A/A, A/G, G/G) were in Hardy-Weinberg equilibrium (χ^2=1.13, p=0.29). The phenotypic effect of heterozygosity (A/G genotype) was intermediate between major (A/A) and minor (G/G) allele homozygotes, indicating that an additive genetic model was appropriate (see below).

Pleiotropic effects of *SGCD* genetic variation on multiple human traits

An additive genetic model for *SGCD* diploid genotype effects on traits demonstrated pleiotropic effects on multiple phenotypes (Table S3, Figure 3), and the effects were confirmed by FDR procedures. The variant/minor G allele, heterozygosity (G/A) or homozygosity (G/G), influenced several biochemical, physical or cardio-metabolic traits: BMI, brachial artery compliance (BAC), SVC, plasma $CHGB_{439\text{-}451}$, pFFA, plasma and urinary (excreted) catecholamines, urine/plasma uric acid ratio, and uric acid in plasma (Table S3).

The *SGCD* peak genetic variant's (rs1835919) directionally coordinate effects on plasma and urine NE are depicted in Figure 3b. The most parsimonious explanation is that the minor allele G gives rise to higher circulating NE, which in turn is excreted, raising urine NE. However, genetic variation *inversely* affected pNE and SVC: as genotype shifted from major allele homozygotes (A/A) through A/G heterozygotes to G/G minor allele homozygotes, pNE increased while SVC decreased (Figure 3c), suggesting an adverse effect of NE on vascular stiffness. Also as a function of *SGCD* genotype, increased secretion of NE provoked increased circulating FFA (Figure 3d). Figures 3e and 3f display *reciprocal* trait effects of the *SGCD* genetic variant: with increased pNE, BMI decreases as shown in Figure 3e. Figure 3f indicates, as a function of *SGCD* genotype, urinary uric acid increases as plasma uric acid decreases. The most parsimonious explanation for this occurrence is that elevated NE increases renal tubular secretion of uric acid, in turn lowering plasma uric acid.

The G (minor) allele (by either heterozygosity or homozygosity) elevated secretion of both NE and $CHGB_{439\text{-}451}$ into plasma (Figure 3g), indicating exocytosis as the mechanism of catecholamine release; FDR confirmed the effects.

SGCD genetic variation: Potential mechanisms to influence expression and human traits

Regulated secretory pathway exocytosis—SGCD is one of 4 sarcoglycan apparatus components, contributing to the dystrophin-glycoprotein complex linking the F-actin cytoskeleton with the extracellular matrix. How might perturbation of this complex influence NE secretion? In a catecholaminergic cellular model of the regulated secretory pathway, trafficking into and out of that pathway can be monitored by storage and secretion of an enzymatically “tagged” version of the regulated secretory protein CHGA, with provocation of exocytosis by the potent depolarizing secretagogue Ba^{2+}. When this system is established in PC12 cells, co-expression of SGCD, representing SGCD over-expression, diminished basal secretion of a CHGA-EAP chimera by ~35%, while preserving stimulated secretion, with an overall *enhancement* of “sorting index” (i.e., efficiency of entry into the regulated secretory pathway) for the chimera, by ~21% (Figure 4a).

Transcriptional motifs at the peak—The *SGCD* rs1835919 peak genetic variant is located in a region of conserved sequence homology. Computationally, the local region encompassing rs1835919 displays a 10/12 bp match for the Hepatocyte Nuclear Factor 3-Beta (HNF3B; aka Forkhead box protein A2 [FOXA2]) transcriptional motif (Figure 4b),

with disruption of the motif by the minor allele. In addition, the more extended local region around rs1835919 harbors additional transcriptional and epigenetic motifs, as demonstrated by Chip-Seq and archived in the ENCODE elements at <genome.ucsc.edu/ENCODE>.

Chromatin Conformation Capture ("Hi-C") domain across *SGCD*—Figure 4c illustrates the local Chromatin Conformation Capture domain structure in the region of rs1835919, with data derived from human embryonic stem cells and IMR90 fibroblasts, and analysis accomplished at <http://chromosome.sdsc.edu>. The plot indicates that the local transcription factor protein/protein interaction domain accessible to the rs1835919 region includes the entire *SGCD* gene, in both the longer (uc0031wa.1) and RefSeq (NM_000337) versions.

***cis*-eQTL at *SGCD*—**Documentation that *cis*-genetic variation at/near the human *SGCD* locus can influence *SGCD* transcript expression is also available as an *SGCD* eQTL at <http://eqtl.uchicago.edu/>, determined for *SGCD* variant rs140615 (p=3.08E-10, with Bonferroni threshold p=3.95E-08), in a dataset coupling expression levels of 39,280 transcripts with 782,476 genotyped SNPs in 427 human liver samples (Schadt *et al.* 2008). *SGCD* variant rs140615 lies in intron-3 of RefSeq *SGCD* isoform-1 gene NM_000337.

DISCUSSION

Overview

A principal finding of our study is that human NE secretion is under substantial genetic control, including a major contribution by the *SGCD* locus on chromosome 5q33, with a relatively common minor allele frequency of the trait-associated variant at ~19%. In carriers of the trait-associated allele, plasma elevations of both NE and CHGB suggest exocytosis as the mechanism of induced NE release, a process that can be directly perturbed by SGCD expression. Thus, not only does rare Mendelian variation at *SGCD* yield human disease, but even common variation gives rise to phenotypic consequences, albeit milder, and without apparent myocardial involvement.

Heritability and pleiotropy in twins

By variance components in twin pairs, we found that the heritability (Table S1) of NE secretion is substantial (at $h^2 = 65.2 \pm 5.0\%$, P=3.19E-16) and the trait correlates with hemodynamic/autonomic and biochemical phenotypes in addition to cardio-metabolic and physical traits, many of which contribute to the metabolic syndrome. Circulating NE increased with age and was higher in men than women. Additionally, we found participants with higher pNE levels showed significant differences in other adrenergic traits, such as increased BP and hypertension risk factors.

Once again, trait-on-trait correlations could be decomposed into shared genetic (RhoG, pleiotropy) versus environmental (RhoE) determination, by variance components (Table S1). Inspection of the genetic correlations in the biochemical realm revealed pleiotropy of pNE with urine NE and plasma CHGB, suggesting exocytosis as the mechanism of catecholamine release. Other genetically co-regulated metabolic traits with pNE included renin secretion. Genetically co-regulated physiological traits included BMI and eGFR. Among cardiovascular traits, SVC displayed pleiotropy with pNE.

Role of SGCD

Mendelian cardiomyopathy in the SCH results from a naturally occurring major deletion in the *SGCD* gene (Nigro et al. 1997). In the SCH, *SGCD* affects not only cardiac, but also skeletal muscle (Nigro et al. 1997, Vainzof *et al.* 2008). Hamsters born with the *SGCD*

variant, and consequent *SGCD* mRNA and protein deficiency, experience a pleiotropic spectrum of cardiac pathologies: cardiac myolysis, hypertrophy and dilation, congestive failure, as well as focal lesions with calcification, necrosis, and fibrosis in the left ventricle as early as 30 to 40 days of life (Nigro et al. 1997, Vainzof et al. 2008, Yamada et al. 1997).

SCH circulating NE levels exceed those of controls as the animals age. Elevated sympathetic tone in rodents and humans can augment or even provoke cardiac pathology (Yamada et al. 1997). Correspondingly, previous studies of patients suffering from chronic heart failure documented the adverse effects of increased levels of circulating NE, and the corresponding therapeutic benefits of adrenergic blockade in patients, particularly with beta-adrenergic antagonists (Schroeder & Jordan 2012).

However, on examination of our subjects, including those with *SGCD*-associated pNE elevation, intrinsic cardiac dysfunction was not observed (Table S3), with the exception of a ~6% decline in SVC (i.e., a modest increment in vascular stiffness), even in the absence of elevated BP. Given that even risk allele homozygotes (G/G) displayed essentially normal mean BP values (127.2±2.9 / 76.0±0.5 mmHg), the pertinence of such modest SVC increments for disease risk is uncertain.

Cell and molecular biology of catecholamine secretion

How might quantitative changes in SGCD perturb NE release or its exocytotic process (suggested by co-release of CHGB, Table S3)? Here we turned to a secretory model of functional catecholaminergic cells, in which we expressed SGCD and then evaluated consequences on exocytotic release of a CHGA-EAP chimera. Indeed, co-expression of SGCD diminished basal secretion of a CHGA-EAP chimera by ~35%, while preserving stimulated secretion, with an overall *enhancement* of the chimera's "sorting index", by ~21% (Figure 4a). Thus, SGCD is sufficient to perturb exocytosis substantially, tightening the trafficking of the chimera into the regulated pathway. While SGCD is primarily known for expression in the myocardium, a variety of sources document its physiological (or endogenous) expression in neuronal and endocrine cells <http://www.ncbi.nlm.nih.gov/geo>, and indeed in PC12 cells used in this experiment (NCBI-GEO dataset GSE-37564), as well as healthy adrenal medulla (NCBI-GEO datasets GDS-3777, GDS-3779). Effects of SGCD on protein trafficking are already documented in the myocardium (Heydemann *et al.* 2007), in that the S151A *SGCD* dominant negative mutation seems to yield protein trafficking defects that disrupt nuclear localization of lamin A/C, emerin, and membrane sarcoglycan.

Do transcriptional alterations underlie the mechanism by which *SGCD* variation changes expression? Using bioinformatic approaches, we were able to show that the peak trait-associated variant disrupts a potential transcription factor binding motif (Figure 4b), and that local patterns of chromosomal conformation yield the potential for the trait-associated region in *SGCD* intron-4 to interact with other domains across the locus (Figure 4c).

Trait-on-trait aggregation: Quantiles

Another factor correlating with the rise in circulating NE, documented previously (Steinberg *et al.* 1964), is the increased circulation of FFA. Steinberg et al. reported that catecholamines, specifically NE, stimulated the mobilization of triglyceride deposits into FFA. Increased FFA, even independent of NE, was found in subjects with high BP, as an indication of sympathetic activity.

Advantages and limitations of this study

We describe an initial link between *SGCD* and sympathetic activity in human subjects, and then document functional connections between SGCD and the exocytotic secretory process. In addition, the evaluation of twins and their families permits us to also determine the heritability of traits to then assess the proportion of phenotypic variation that is attributable to genetic variation. Whether our results in predominantly healthy individuals can be extrapolated to disease populations is not yet certain.

CONCLUSIONS AND PERSPECTIVES

A schematic formulating our results into a global hypothesis is presented in Figure 4d, outlining the role of intermediate phenotypes and the *SGCD* gene influence on sympathetic and cardio-metabolic traits. By focusing on naturally occurring genetic variation at *SGCD*, we discovered a novel determinant of human NE secretion. Similar SGCD effects were also observed on a number of biochemical, physical, and cardio-metabolic traits; indeed, coordinate elevation of NE and CHGB suggested exocytosis as the mechanism of co-release, a process that could be directly perturbed by SGCD expression. However, in our predominantly healthy subject group, common variation at *SGCD* exhibited no evidence of myocardial pathology. Plasma NE concentration and several of the elevated physical and cardio-metabolic traits are highly heritable and known cardiovascular risk factors; secreted NE displayed joint genetic determination (pleiotropy) with hypertension risk factors such as the other catecholamines (DA and EPI) and SVC. Our results document a novel *trans*-QTL for sympathetic activity, in addition to suggesting new possibilities for its regulation. Further investigation may confirm the current results and establish specific molecular mechanisms for human adrenergic trait variation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (NHLBI and NIDDK), and the Department of Veterans Affairs.

GLOSSARY OF ABBREVIATIONS

BAC	Brachial artery compliance
BMI	Body mass index
BP	Blood pressure
BSA	Body surface area
CHGA	Chromogranin A
CHGB	Chromogranin B
ChIP-seq	Chromatin immunoprecipitation sequencing
CKD-EPI algorithm	Chronic Kidney Disease EPIdemiology collaboration algorithm
COMT	catechol-O-methyltransferase
CSPD	3-(4-methoxyspiro [1,2-dioxetane-3,2-(5chloro)tricyclo[3.3.1.1(3,7)]decan] 4-yl) phenyl phosphate

DA, pDA	Dopamine, plasma dopamine
DGC	Dystrophin-glycoprotein complex
DPB	Diastolic blood pressure
DZ	Dizygotic
EAP	Embryonic alkaline phosphatase
eGFR, eGFR	Estimated glomerular filtration rate
EPI, pEPI	Epinephrine, plasma epinephrine
eQTL	Expression quantitative trait loci
FDR	False Discovery Rate
FFA, pFFA	plasma free fatty acids
GEE	Generalized estimating equations
GWAS	Genome-wide association study
HiC	Chromatin Conformation Capture
MZ	Monozygotic
NE, pNE	Norepinephrine, plasma norepinephrine
NPY, pNPY	Neuropeptide Y, plasma neuropeptide Y
QUICKI	QUantitative Insulin sensitivity ChecK Index
RefSeq	Reference sequence
RhoG (ρ_G)	Genetic covariance
RhoE (ρ_E)	Environmental covariance
SBP	Systolic blood pressure
SCH	Syrian Cardiomyopathic Hamster
SEAP	Secreted embryonic alkaline phosphatase
SGCD	Delta-sarcoglycan
SNAP	SNP Annotation and Proxy Search
SNP	Single nucleotide polymorphism
SOLAR	Sequential Oligogenic Linkage Analysis Routines
SVC	Systemic vascular compliance
SVR	Systemic vascular resistance

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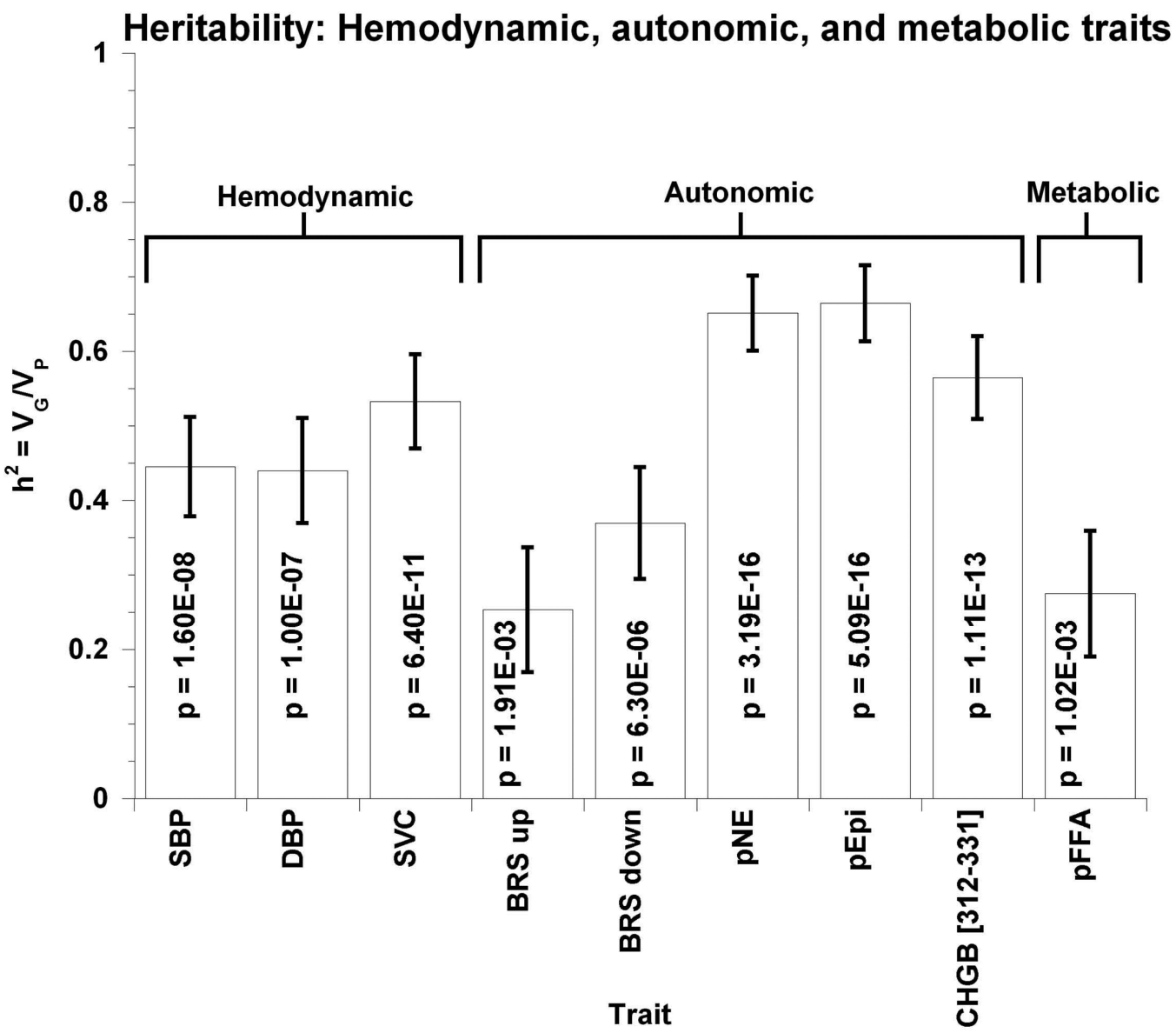


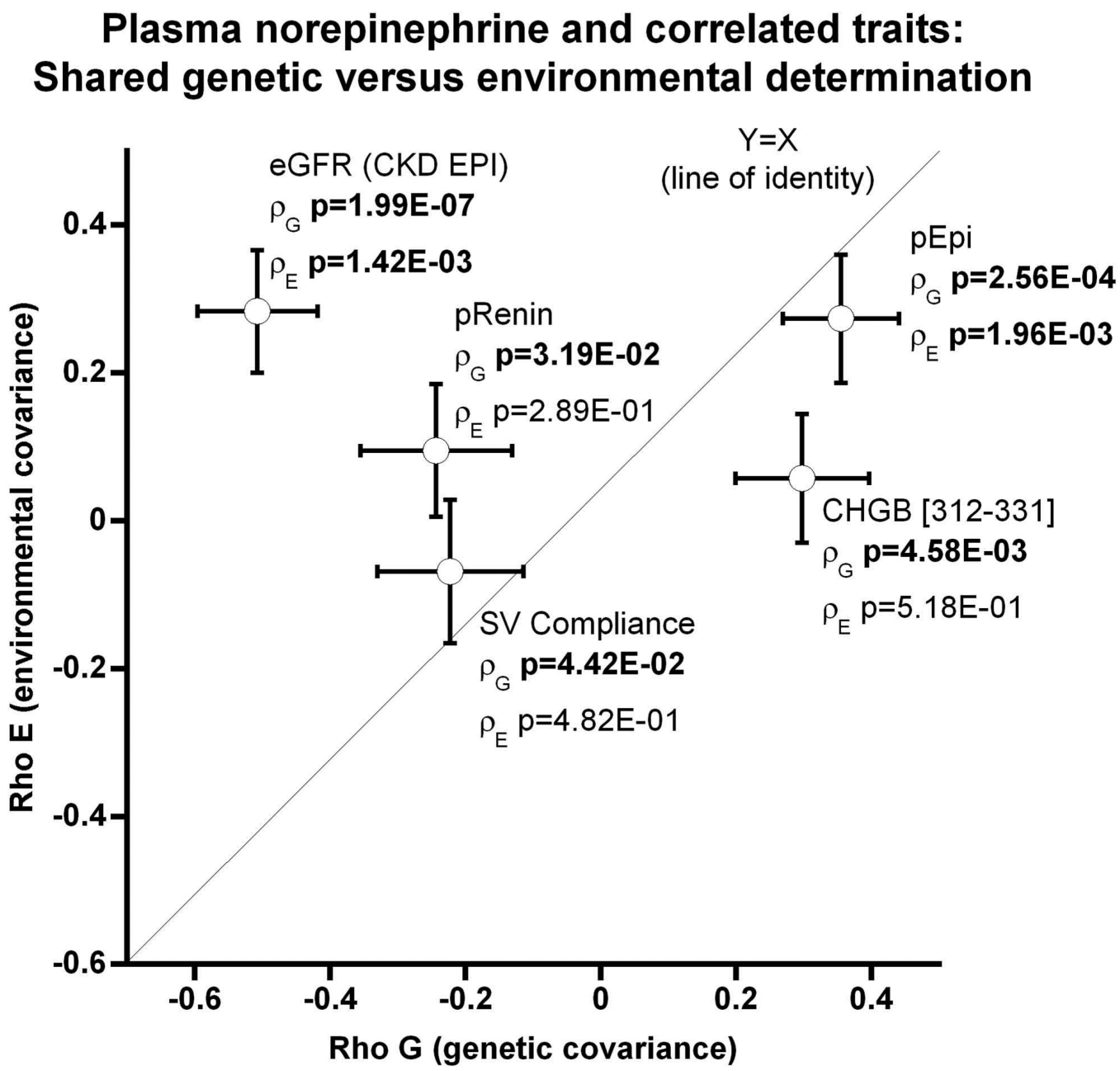
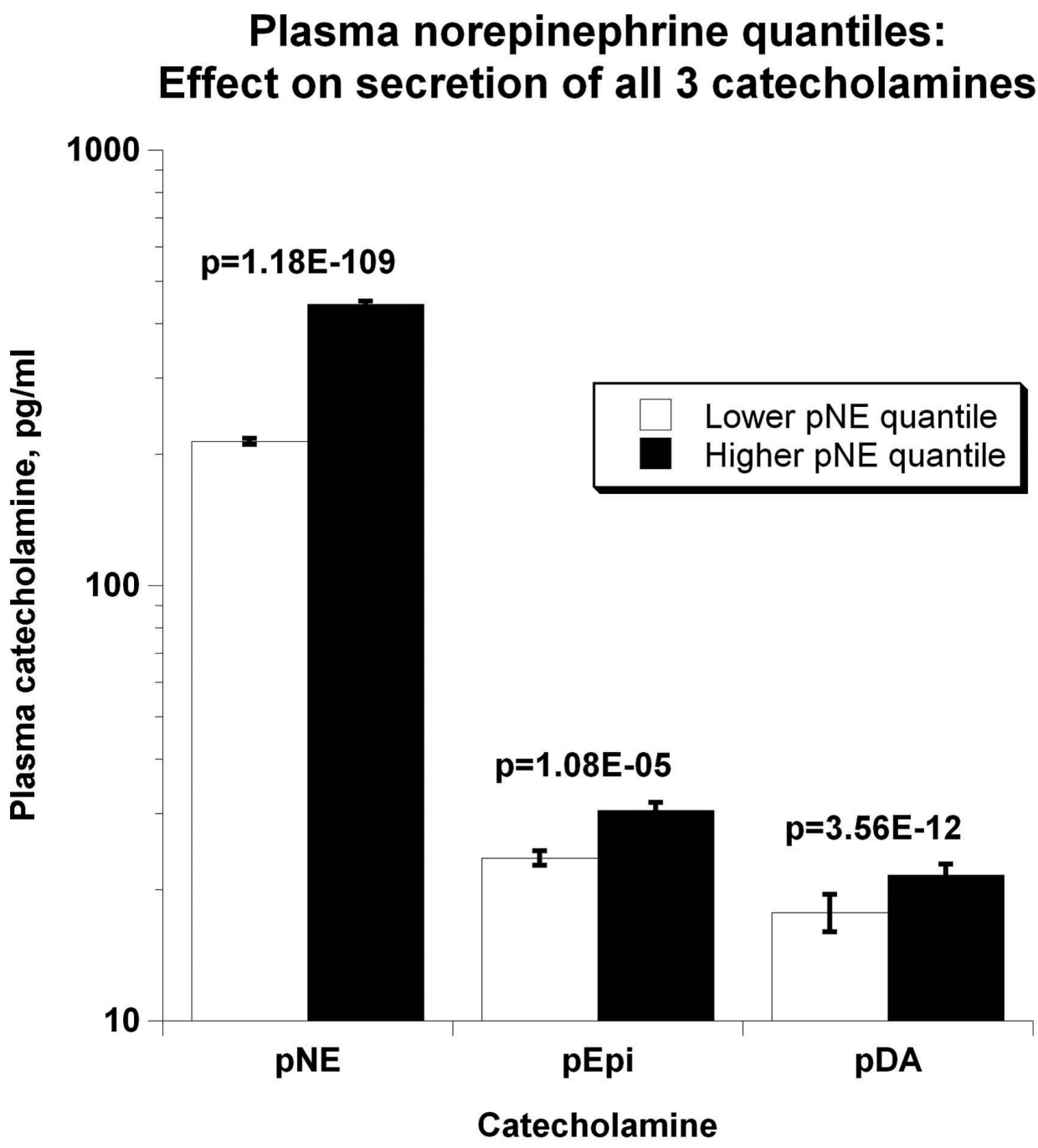


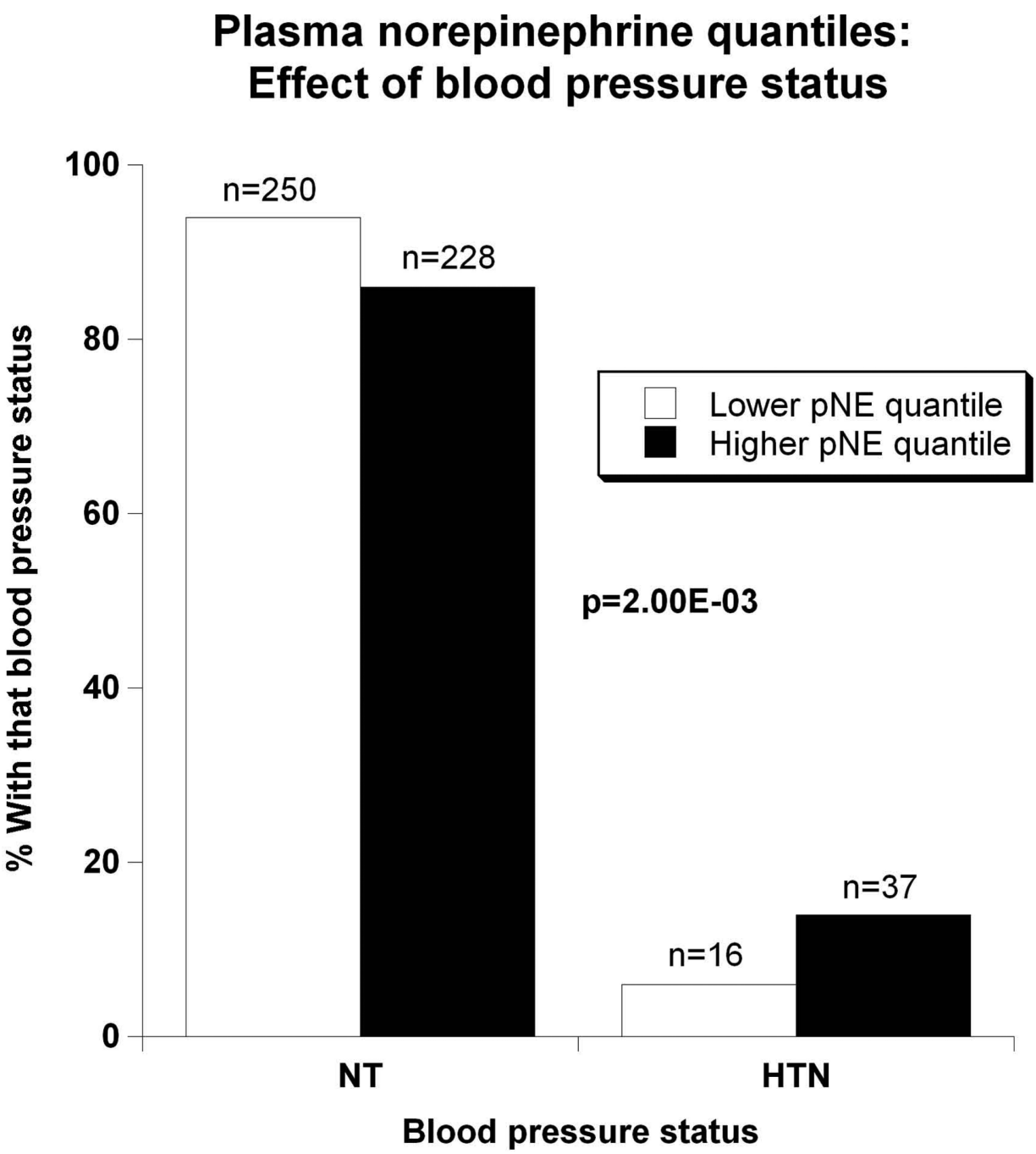
Figure 1. Trait determination: Relative roles of heredity and environment, estimated by variance components in twin pairs

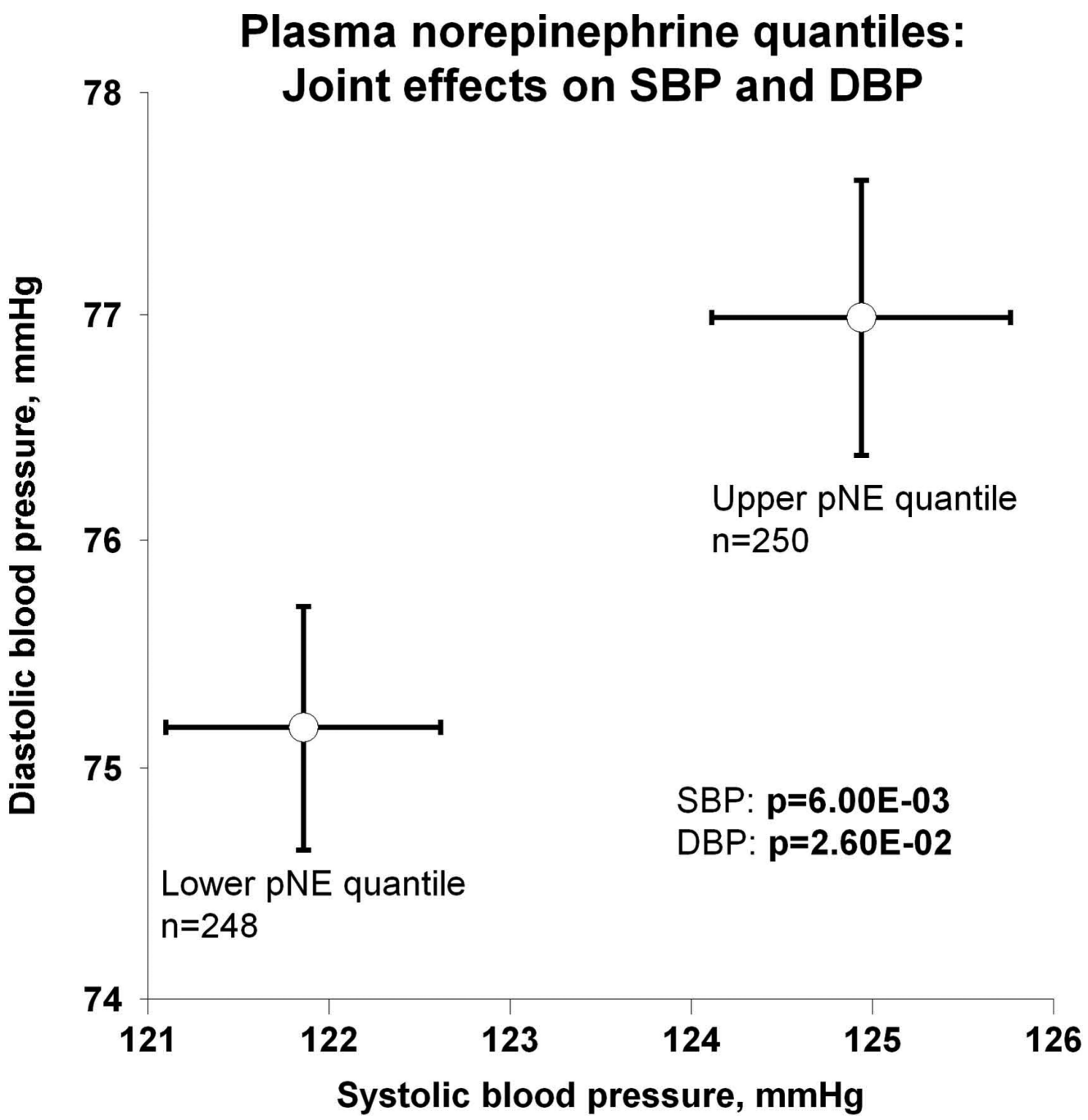
Results are shown as the mean ± SEM for traits. Significant differences (P<0.05) are **bold**. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; SVC/SV Compliance, systemic vascular compliance; BRS up, baroreceptor slope upward deflection, BRS down, baroreceptor slop downward deflection; pEPI, plasma epinephrine; CHGB, chomogranin B; pFFA, plasma free fatty acids; eGFR (CKD-EPI), estimated glomerular filtration rate by Chronic Kidney Disease Epidemiology Collaboration algorithm; pRenin, plasma active renin.

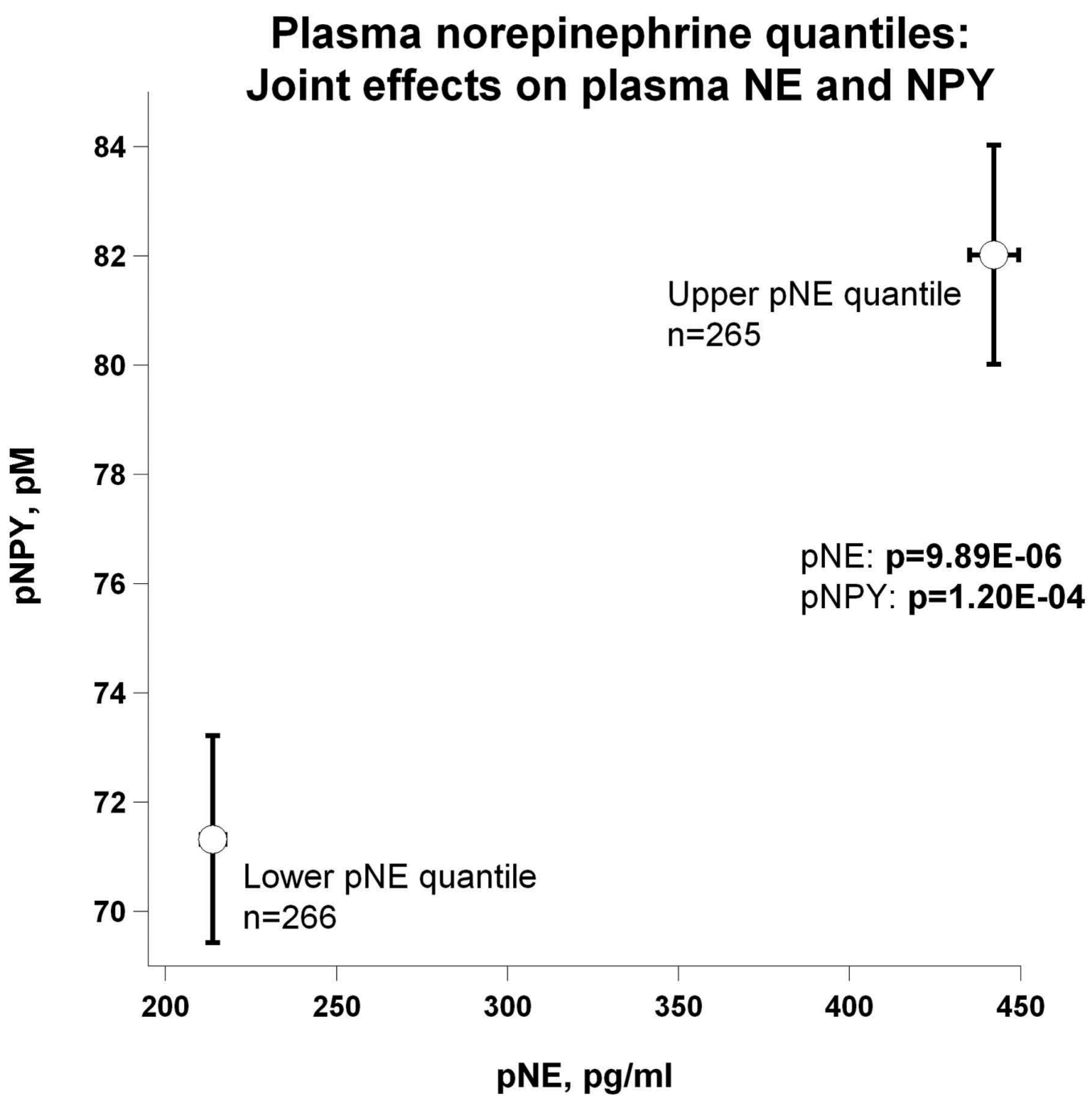
(**a**) Heritability (h^2) of hemodynamic, autonomic, and metabolic traits.

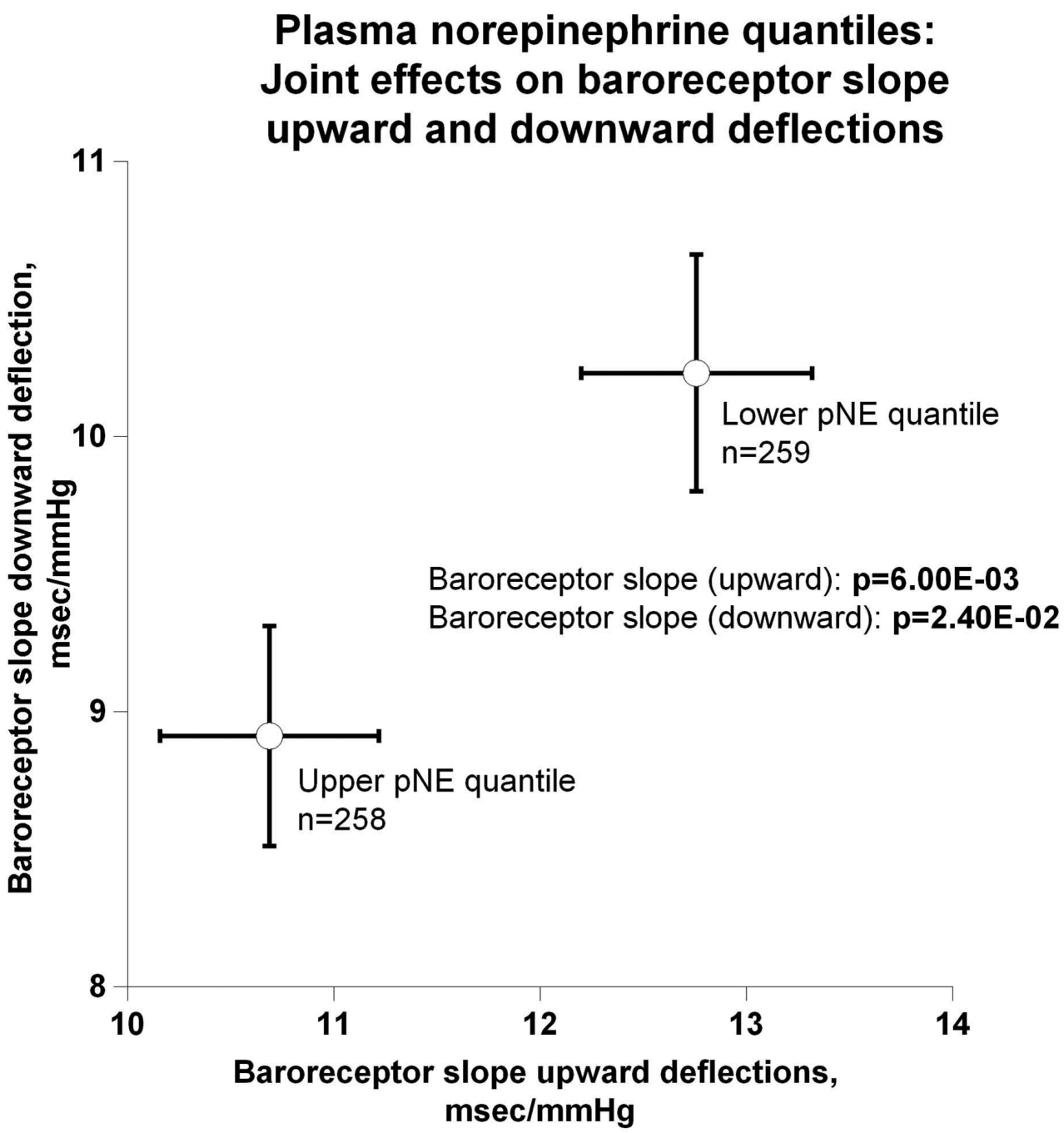
(**b**) Genetic pleiotropy for plasma norepinephrine (pNE) with correlated traits, plotting RhoG (genetic covariance) as a function of RhoE (environmental covariance). The diagonal line is the theoretical line of identity ($Y = X$).

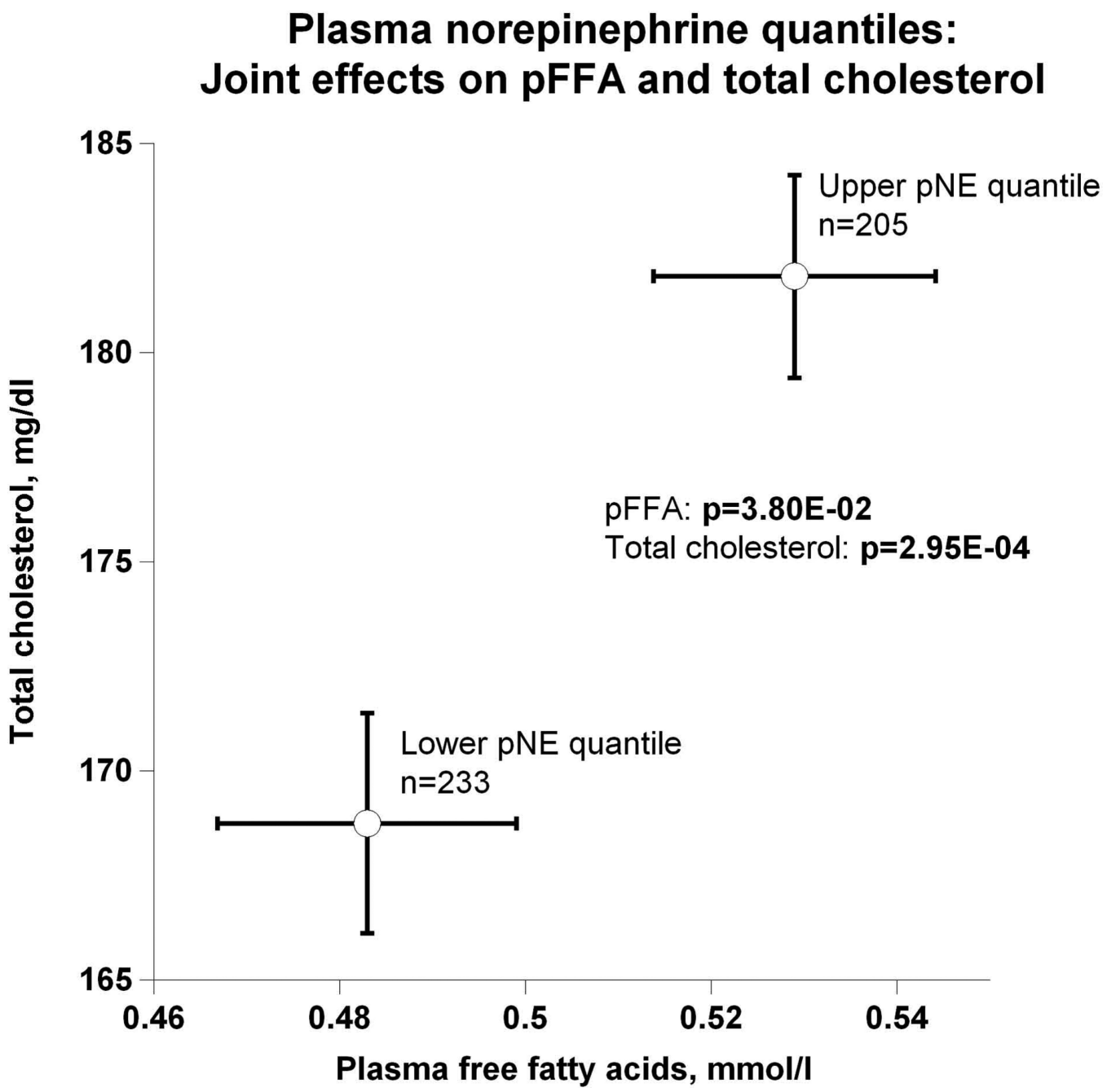












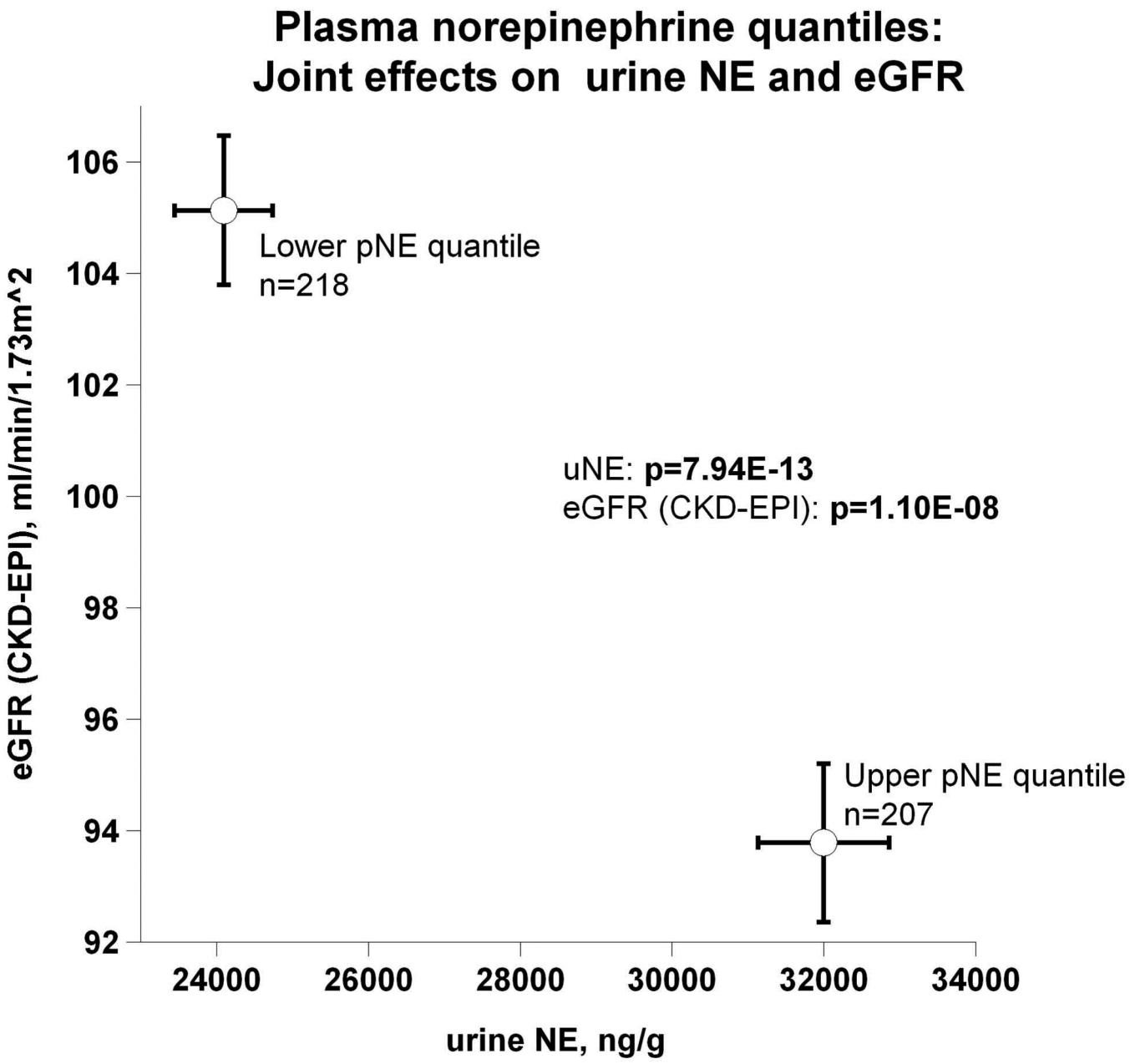


Figure 2. Trait aggregation of plasma norepinephrine (pNE) with physiological traits

Results (shown as mean ± SEM) were analyzed by independent sample t-test. Significant differences (P<0.05) are **bold**.

(a) Effects on the secretion of all three catecholamines pNE, epinephrine (pEPI), and dopamine (pDA);

(b) Effects on blood pressure status (NT indicates normotensive; HTN, hypertensive);

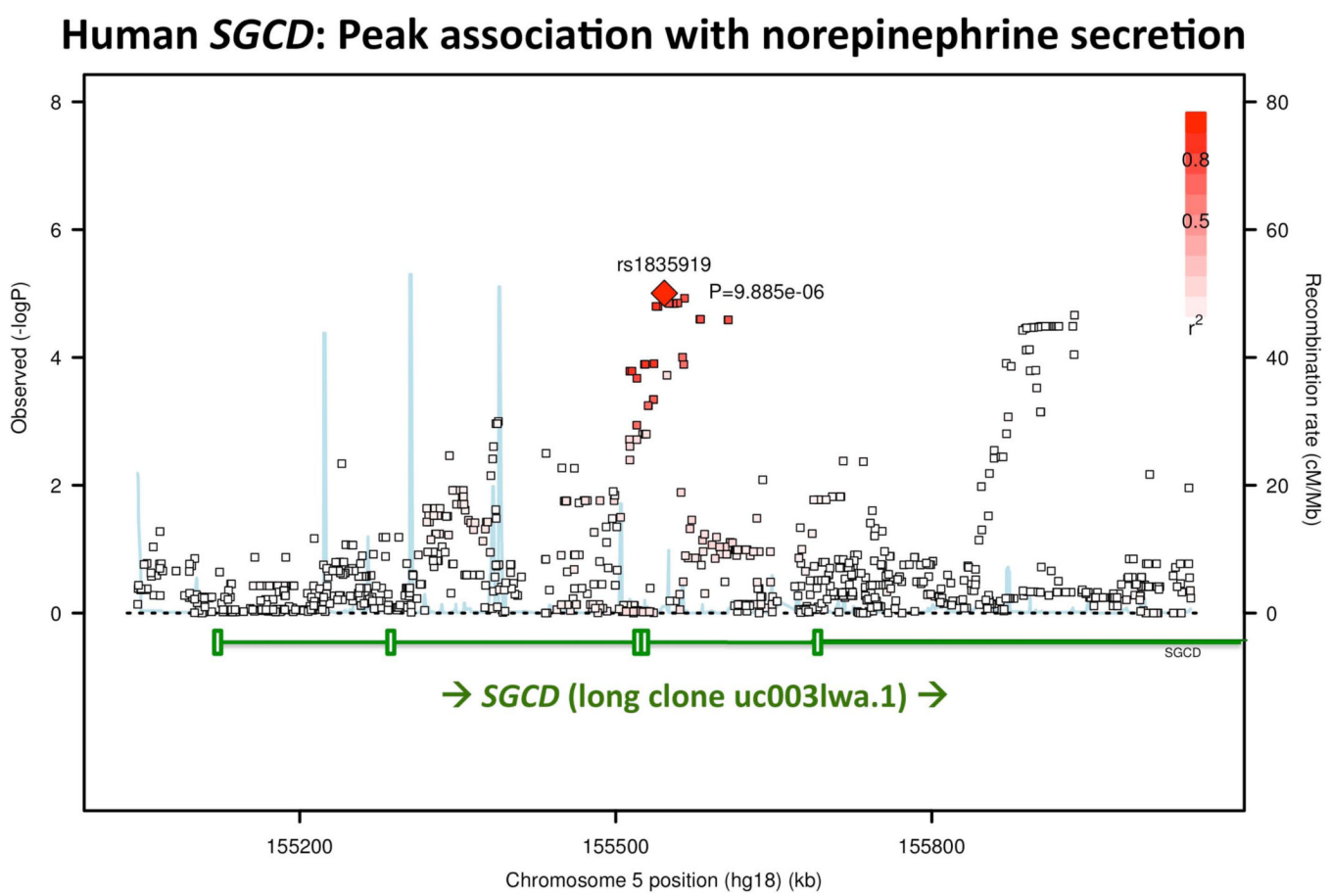
(c) Joint effects on systolic (SBP) and diastolic blood pressure (DBP);

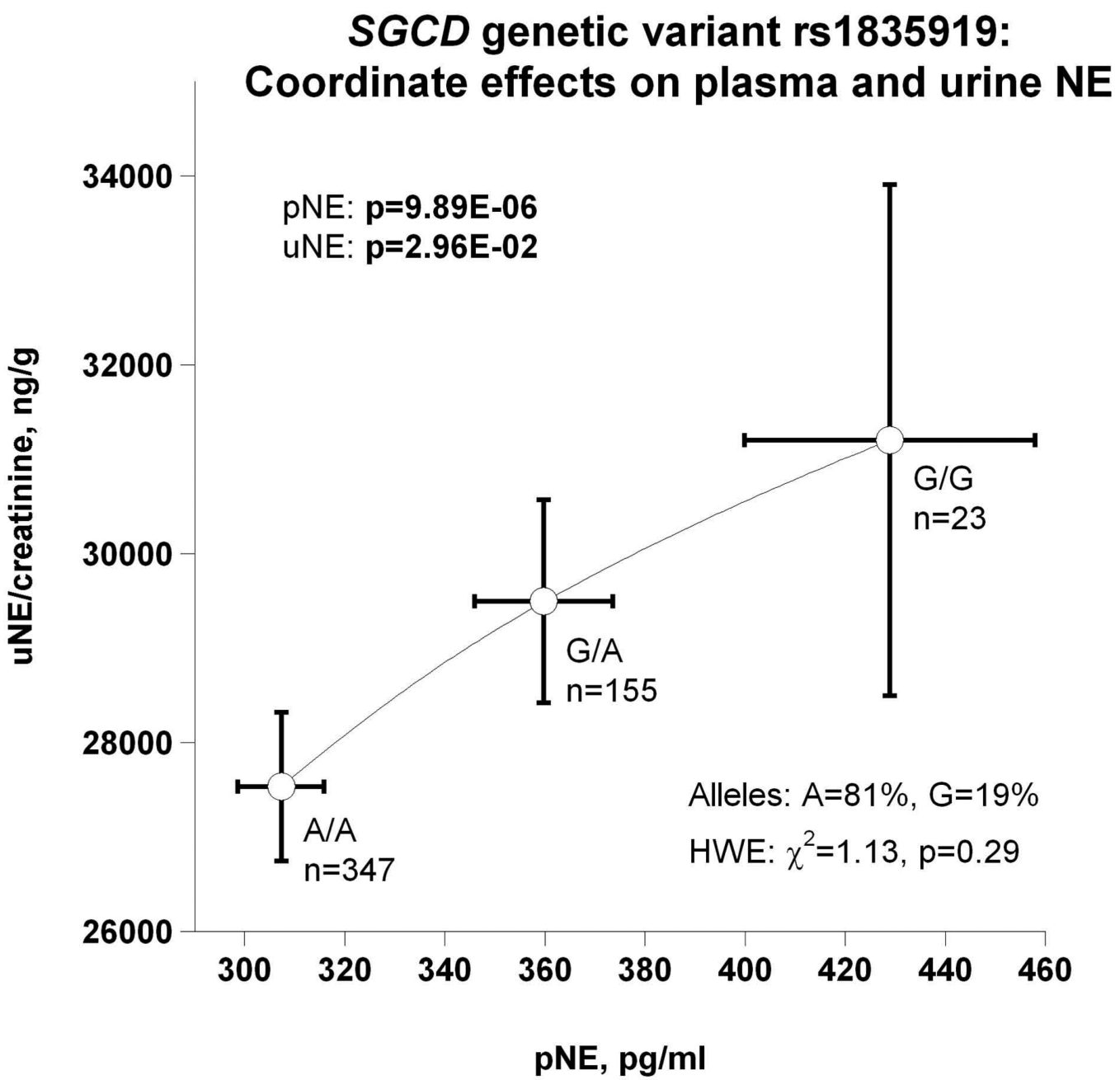
(d) Joint effects on pNE and plasma neuropeptide Y (pNPY);

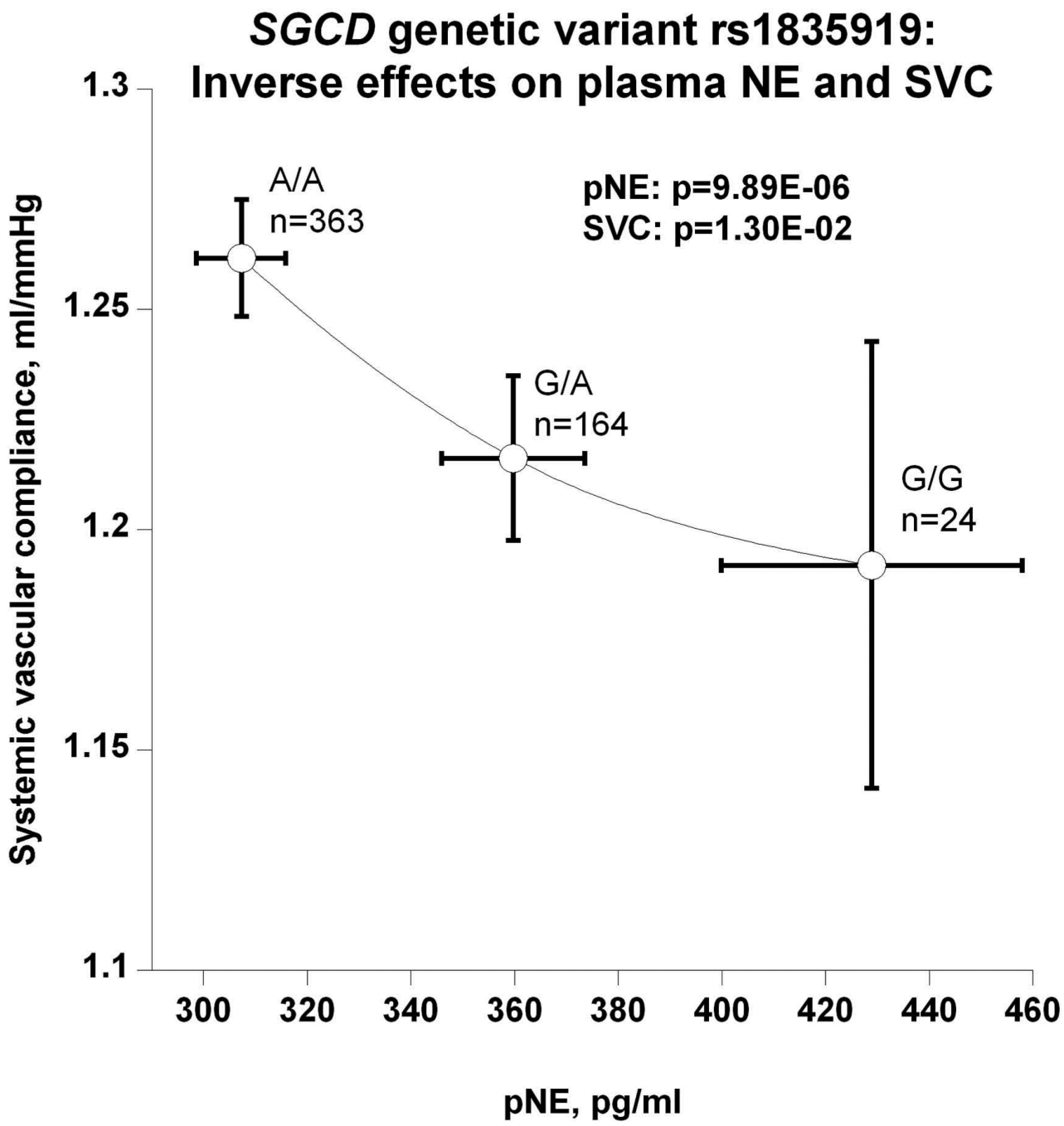
(e) Joint effects on baroreceptor slope upward and downward deflections;

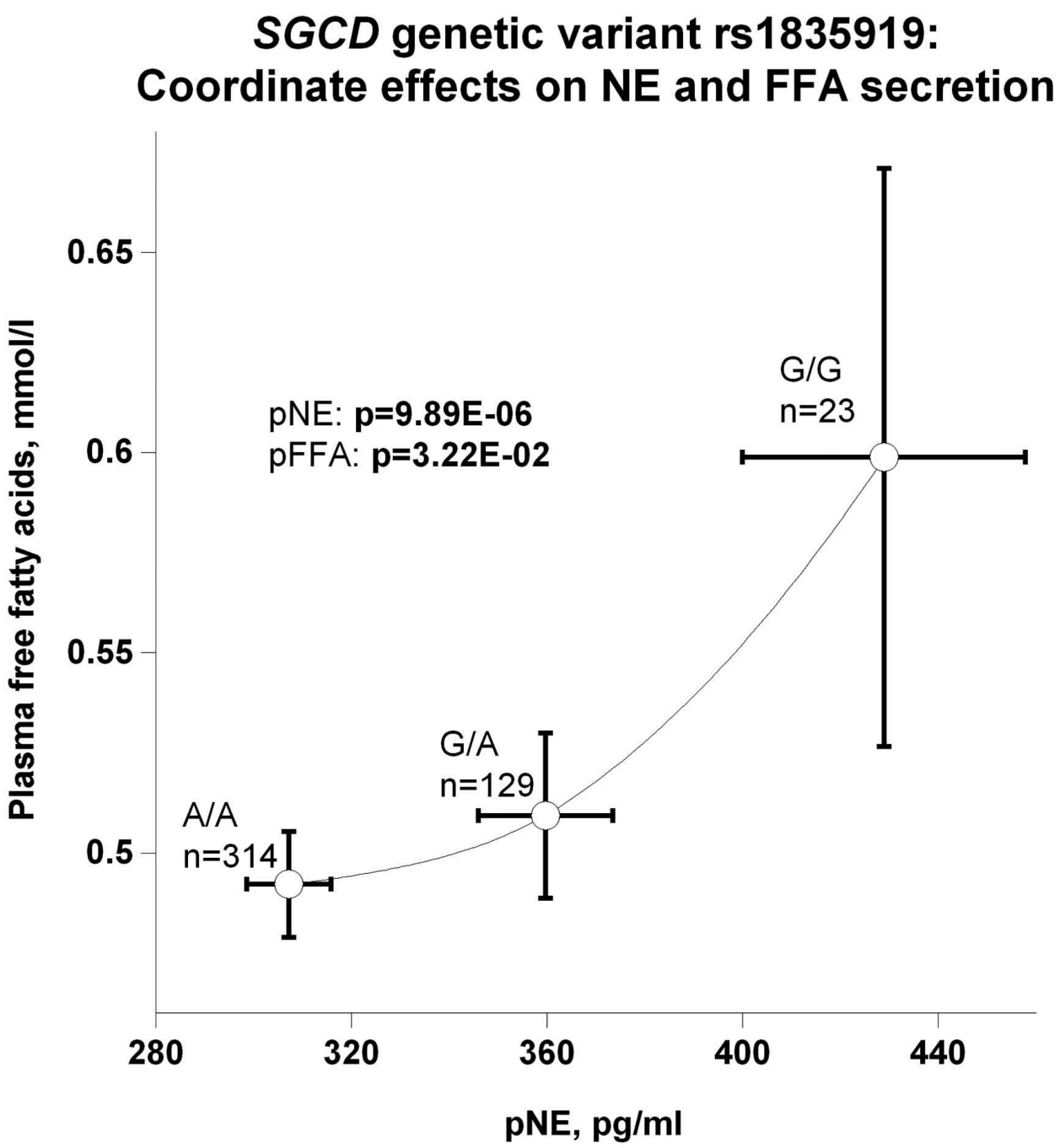
(f) Joint effects on plasma free fatty acids (pFFA) and total cholesterol;

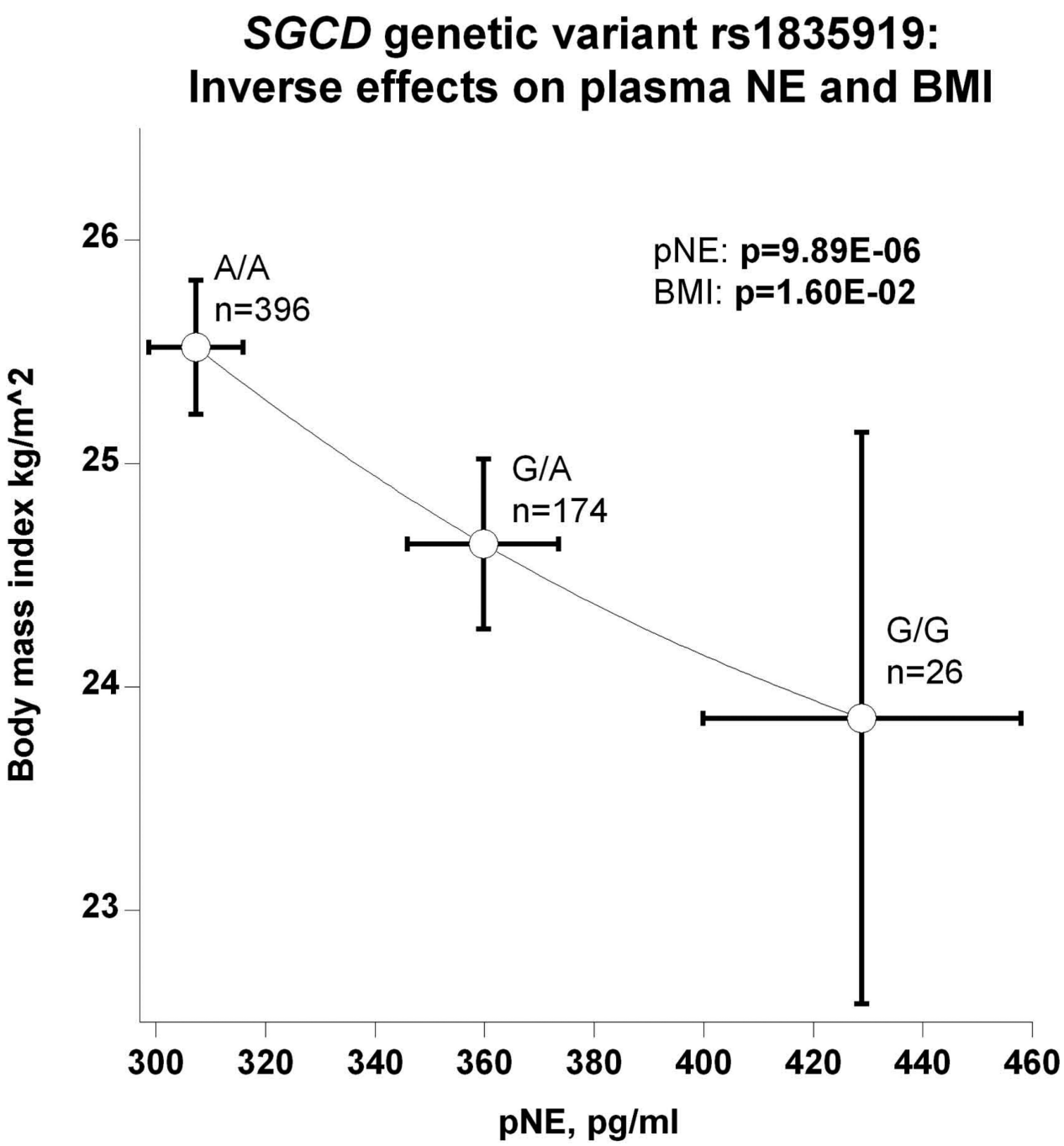
(g) Joint effects on urine norepinephrine (uNE) and estimated glomerular filtration (eGFR) rate (by CKD-EPI, Chronic Kidney Disease EPIdemiology collaboration algorithm).

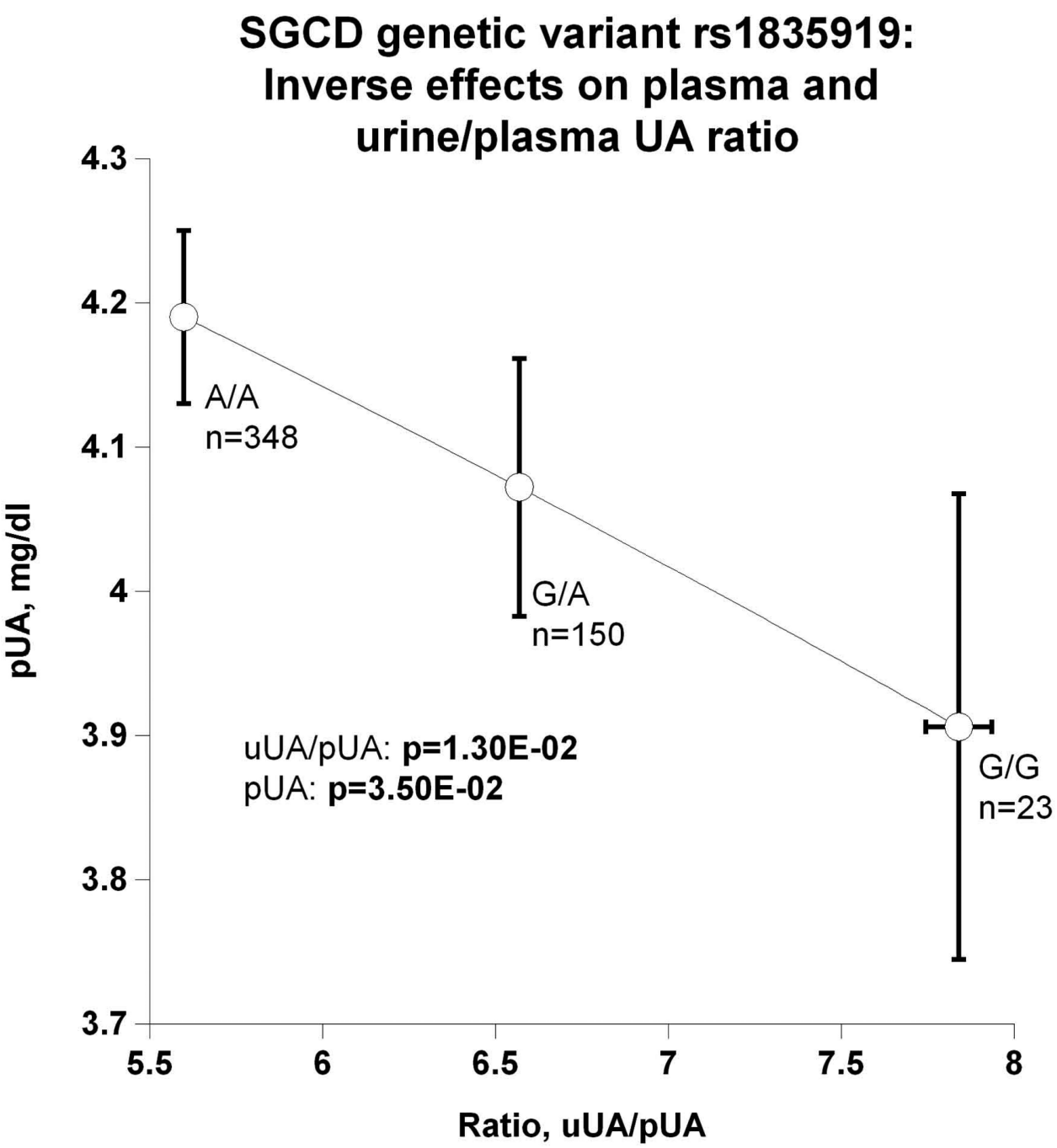












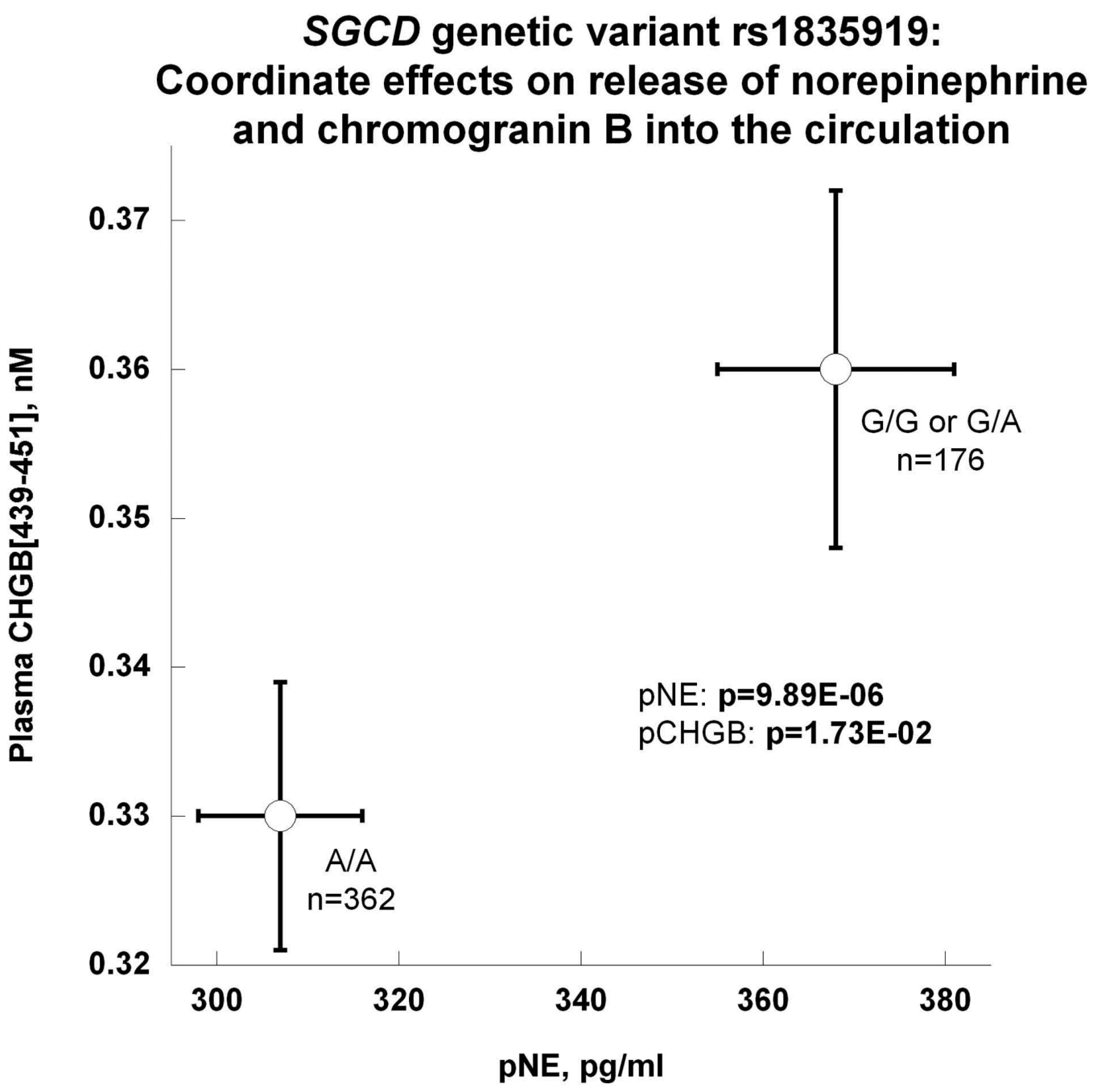


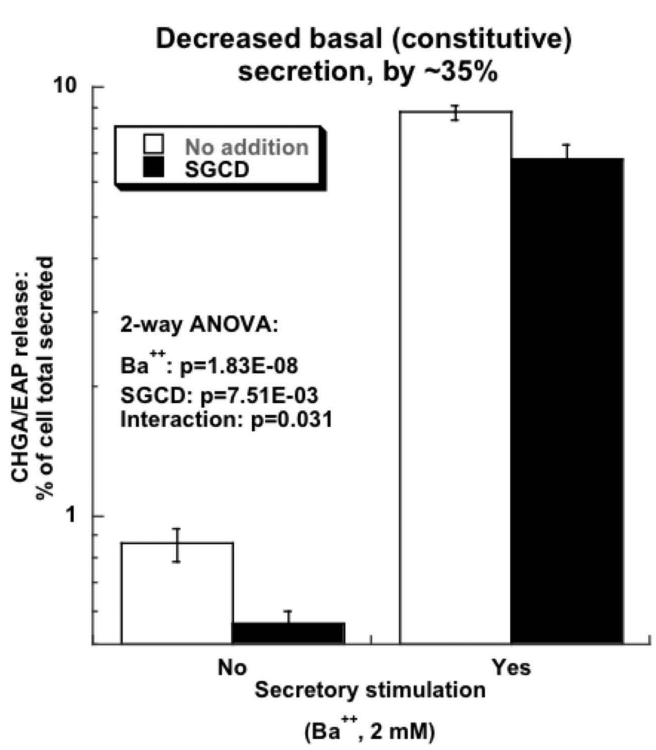
Figure 3. Genetic determination of traits by variation at the delta-sarcoglycan (*SGCD*) locus: Effects upon norepinephrine (NE) secretion as well as other physical, physiological, and biochemical traits

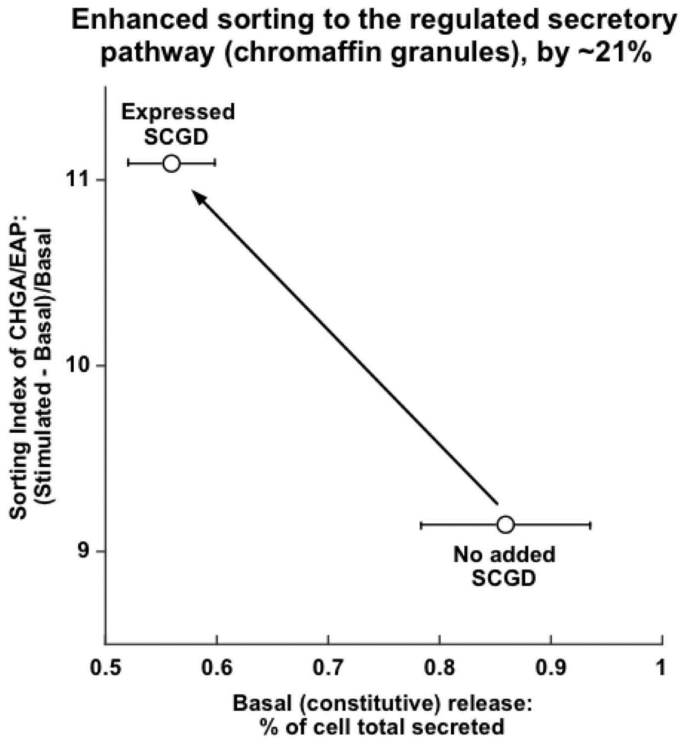
Results are shown as mean ± SEM. Significant differences (P<0.05) are **bold**.

(**a**) Peak effect in the *SGCD* local region on chromosome 5q33. Local region of the Manhattan plot displayed by SNAP (SNP Annotation and Proxy Search) plot <http://www.broadinstitute.org/mpg/snap/ldplot.php>. The peak associated with NE secretion is at *SGCD* (intron-4, rs1835919, in long clone uc0031wa.1).

(**b**) Coordinate effects on plasma and urine NE (uNE indicates urine norepinephrine; uUA urine uric acid, pUA, plasma uric acid; associated allele frequency and Hardy-Weinberg equilibrium (HWE) test results displayed);
(**c**) Inverse effects on plasma NE (pNE) and systemic vascular compliance (SVC);
(**d**) Coordinate effects on pNE and plasma free fatty acids (pFFA) secretion;
(**e**) Inverse effects on pNE and body mass index (BMI);
(**f**) Inverse effects on plasma and urine/plasma uric acid (UA) ratio;
(**g**) Coordinate effects on release of NE and chromogranin B (CHGB [439-451]) into the circulation.

Expression of SGCD and exocytosis in PC12 cells: Enhanced efficiency of the regulated secretory pathway



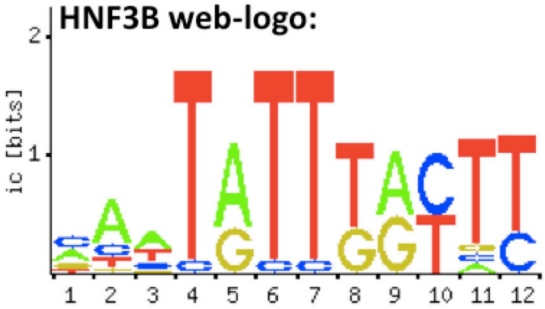


Human *SGCD* non-coding genetic variant rs1835919:

Sequence conservation and disruption of a HNF3B (FOXA2) transcriptional motif

```
Primate species alignment:
Human T_allele     TG-CTTTCTTTAGCTAATATTTGAGGAGGAGAATGATCTC
Human C_allele     TG-CTTTCTTTAGCTAATACTTGAGGAGGAGAATGATCTC
Chimp              T--------------AATATTTGAGGAGGAGAATGATCTC
Orangutan          TG-CTTTCTTTAGCTAATATTTGAGGAGGAGAATGATCTC
Rhesus             TG-CTTTCTTAAACTAATATTTGAGGAGGAGAATGATCTC
Marmoset           TG-CTTTCTTAAGCTAACATTTGAGGAGGCGAATGATCTC
Conservation                          ********** **********
                                           ↑
                                          T/C
```

HNF3B web-logo:



T/C

HNF3B position weight frequency matrix:

A	[	6	11	8	0	11	**0**	0	0	9	0	1	0]
C	[	7	3	3	1	0	**1**	1	0	0	9	1	5]
G	[	3	1	1	0	6	**0**	0	6	8	0	1	0]
T	[	1	2	5	16	0	**16**	16	11	0	8	14	12]

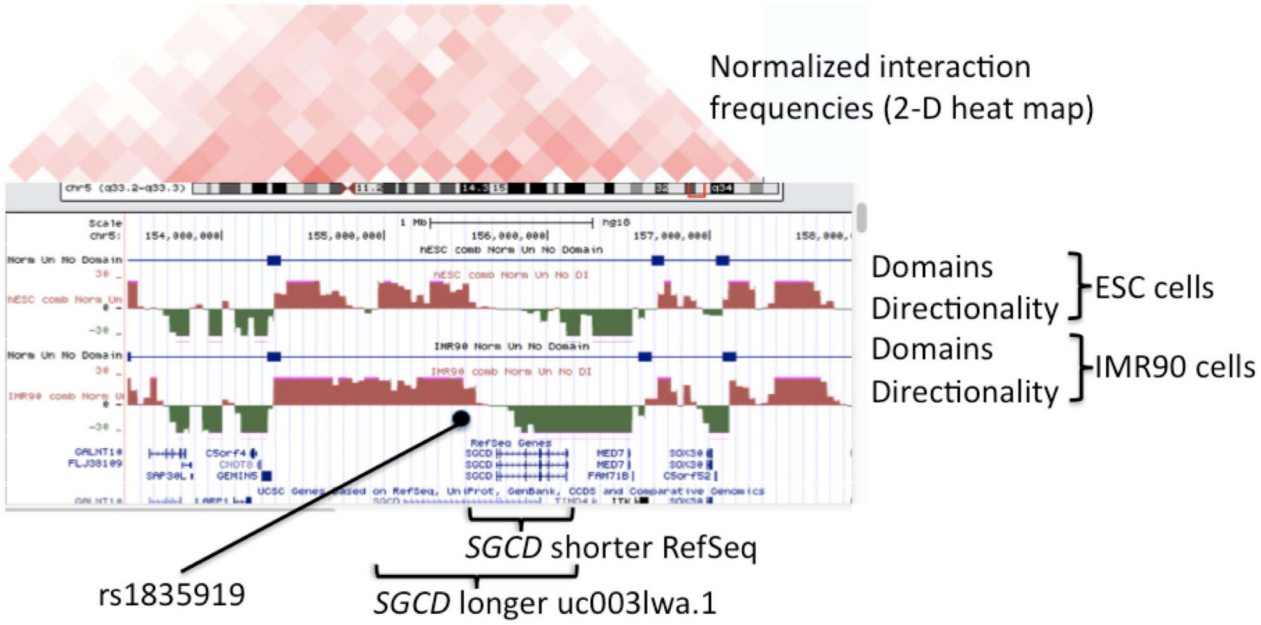
T/C

Sequence alignment at: http://genome.ucsc.edu

Motif alignment of T/C polymorphism on the [-] strand

Motif search at CONSITE: <http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/>

"Hi-C" (chromatin conformation capture) at the human *SCGD* locus on chr-5q33: Local chromatin interaction domains and boundaries



Hi-C analysis at: <http://chromosome.sdsc.edu>

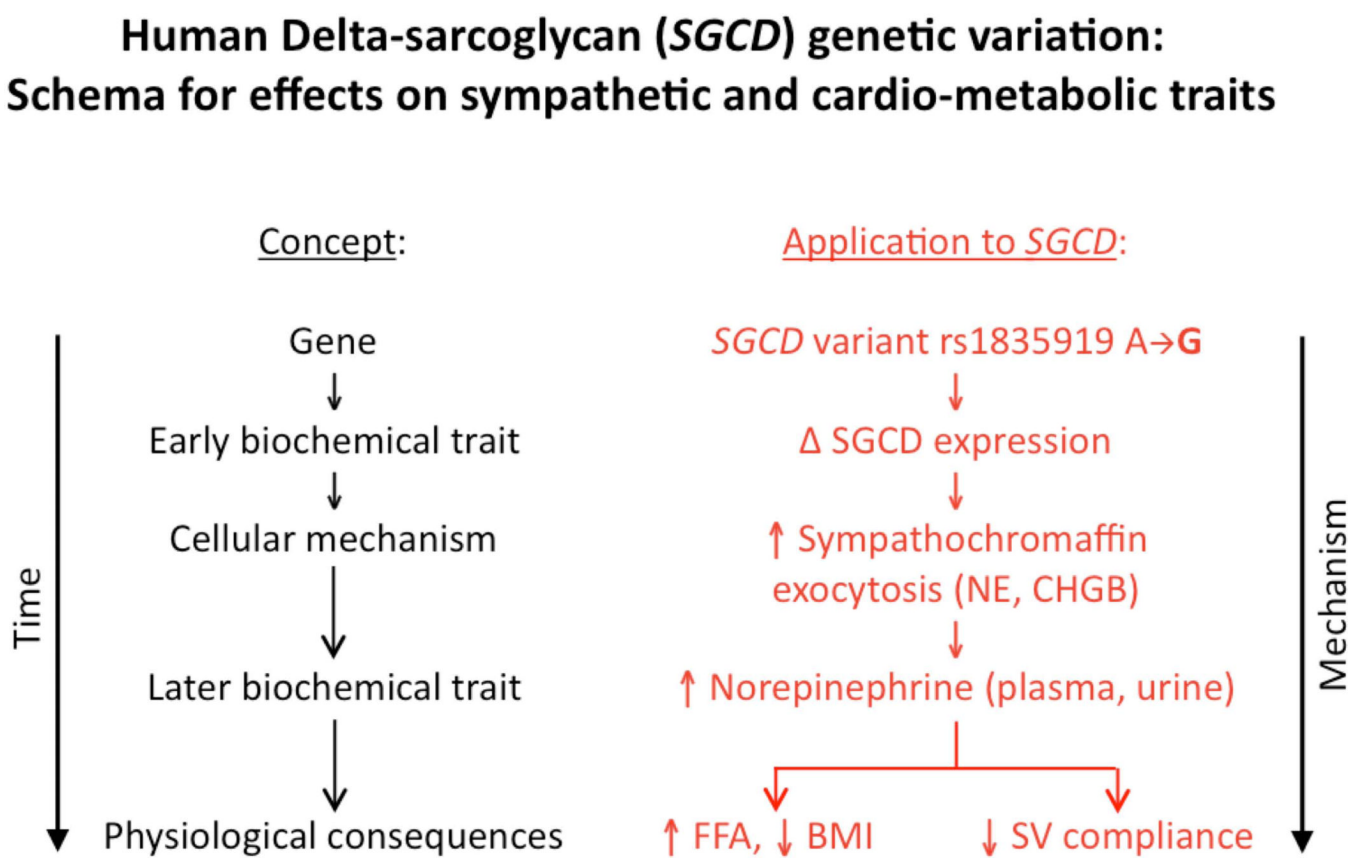


Figure 4. Delta-sarcoglycan (*SGCD*): Potential mechanisms influencing trait variation

Results are shown as mean ± SEM. Significant differences (P <0.05) are **bold**.

(**a**) Protein trafficking into the regulated secretory pathway in PC12 cells, co-transfected with pCMV-SGCD and pCMV-CHGA-EAP chimera. Decreased stimulated secretion of Chromogranin A (CHGA) following SGCD exposure in PC12 cells, with and without secretory stimulation. In this system, co-expression of SGCD enhances CHGA secretory protein sorting to the regulated pathway.

(**b**) Transcription factor binding. The peak associated *SGCD* variant disrupts a potential Hepatocyte nuclear factor 3-beta (HNF3B; aka Forkhead box protein A2 (FOXA2)) transcriptional motif. The *SGCD* transcriptional motif is shown to maintain conservation through inter-species sequence homology; polymorphism in bold, *SGCD* promoter SNP rs1835919 ([-] strand, as T>C). Also displayed is the graphical representation of HNF3B motif multiple sequence alignment and the position frequency and weight matrix representing known variation at the HNF3B alignment.

(**c**) Chromatin Conformation Capture. The plot indicates that the local interaction domain <http://chromosome.sdsc.edu> accessible to the rs1835919 region includes the entire *SGCD* gene, in both the longer (uc0031wa.1) or RefSeq (NM_000337) versions.

(**d**) The schematic presents a hypothetical framework integrating the overall rationale and experimental results of our study, and suggests future questions for exploration. CHGB indicates chromogranin B; all other abbreviations as in Figures 1 and 3. Ba^{2+} indicates barium; EAP, embryonic alkaline phosphatase.