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Circ Cardiovasc Genet 2009;2;46-56; originally published online Jan 23, 2009;
DOI: 10.1161/CIRCGENETICS.108.785659
Circulation: Cardiovascular Genetics is published by the American Heart Association. 7272 Greenville
Avenue, Dallas, TX 72514
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Print ISSN: 1942-325X. Online
ISSN: 1942-3268

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Autonomic Function in Hypertension

Role of Genetic Variation at the Catecholamine Storage Vesicle Protein Chromogranin B

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Background**—**Hypertension is a complex trait, with deranged autonomic control of circulation. Chromogranin B (*CHGB*) is the most abundant core protein in human catecholamine secretory vesicles, playing an important role in their biogenesis. Does common interindividual variation at the *CHGB* locus contribute to phenotypic variation in CHGB and catecholamine secretion, autonomic stability of circulation, or blood pressure (BP) in the population?

Methods and Results**—**To probe interindividual variability in *CHGB*, we systematically studied polymorphism across the locus by resequencing *CHGB* (6 kbp footprint spanning the promoter, 5 exons, exon/intron borders, untranslated regions) in 160 subjects (2n 320 chromosomes) of diverse biogeographic ancestries. We identified 53 single-nucleotide polymorphisms, of which 22 were common. We then studied 1182 subjects drawn from the most extreme BP values in the population (highest and lowest 5th percentiles), typing 4 common polymorphisms spanning the 14 kbp locus. Sliding-window haplotype analysis indicated BP associations peaking in the 5 /promoter region, most prominent in men, and a peak effect in the proximal promoter at variant A-261T (A T), accounting for 8/ 6 mm Hg BP in males. The promoter allele (A-261) that was a predictor of higher diastolic BP and systolic BP was also associated with lower circulating/plasma CHGB concentration (CHGB$_{439\ \mathrm{to}\ 451}$ epitope) in twin pairs. In twins, the same *CHGB* variants that were predictors of lower basal CHGB secretion were also associated with exaggerated catecholamine secretion and BP response to environmental (cold) stress; likewise, women displayed increased plasma CHGB$_{439\ \mathrm{to}\ 451}$ but decreased catecholamine secretion as well as BP response to environmental stress. The effect of A-261T on CHGB expression was confirmed in chromaffin cells by site-directed mutagenesis on transfected *CHGB* promoter/luciferase reporter activity, and the allelic effects of A-261T on gene expression were directionally coordinate in cella and in vivo. To confirm these clinical associations experimentally, we undertook targeted homozygous (/) ablation of the mouse *CHGB* gene; knockout mice displayed substantially increased BP, by 20/ 18 mm Hg, confirming the mechanistic basis of our findings in humans.

Conclusion**—**Common genetic variation at the *CHGB* locus, especially in the proximal promoter, influences *CHGB* expression and later catecholamine secretion and the early heritable responses to environmental stress, eventuating in changes in resting/basal BP in the population. Both the early (gene expression) and late (population BP) consequences of *CHGB* variation are sex dependent. These results point to new molecular strategies for probing autonomic control of circulation and, ultimately, the susceptibility to and pathogenesis of cardiovascular disease states such as hypertension. **(*Circ Cardiovasc Genet*. 2009;2:46-56.)**

Key Words: genetics hypertension gene expression catecholamine epidemiology nervous system autonomic

Hypertension is a complex trait in which deranged autonomic control of circulation may be an early etiologic culprit. The sympathoadrenal system exerts minute-to-minute

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Received April 15, 2008; accepted November 20, 2008.

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The online-only Data Supplement is available at http://circgenetics.ahajournals.org/cgi/content/full/CIRCGENETICS.108.785659/DC1.

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***Circ Cardiovasc Genet* is available at http://circgenetics.ahajournals.org** **DOI: 10.1161/CIRCGENETICS.108.785659**

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control over cardiac output and vascular tone. Genes governing catecholaminergic processes may play a role in the development of hypertension.[1] The chromogranins/secretogranins comprise a family of acidic, soluble proteins that are widely stored in secretory granules with hormones, transmitters, and neuropeptides throughout the endocrine and nervous systems.[2] Chromogranin B (CHGB), first described in the 1980s,[3,4] appears to be the quantitatively most abundant matrix protein in the core of human catecholamine storage vesicles.[5,6] Overexpression and underexpression studies in chromaffin cells indicate that CHGB plays an important role in secretory vesicle biogenesis.[7]

CHGB has both extracellular and intracellular roles in the neuroendocrine system. Extracellular roles for CHGB are dependent on its extensive proteolytic processing within chromaffin granules at dibasic cleavage sites[8] to form smaller peptides; such peptides may have a role in the neuroendocrine/sympathoadrenal stress response to systemic infection.[2] Within chromaffin cells and sympathetic axons, CHGB functions in sorting and trafficking of peptide hormone and neuropeptide precursors to secretory granules,[9] perhaps as triggers to secretory granulogenesis.[7]

Because excess sympathetic activity is implicated in causing hypertension,[10] and alterations in sympathetic responses may occur even in the normotensive relatives of patients at genetic risk for later development hypertension, we wondered whether the sympathochromaffin mechanisms, such as the catecholaminergic CHGB system, might be altered in hypertension or in individuals at risk for development of hypertension. In this study, we undertook a systematic study of polymorphism at the human locus, by resequencing *CHGB*, discovering and associating a series of naturally occurring *CHGB* variants with gene expression in vivo, and series of sympathochromaffin traits eventuating in the disease state of essential hypertension.

Methods

Subjects and Clinical Characterization

Subjects were volunteers from urban southern California (San Diego), and each subject gave informed, written consent; the protocol was approved by the institutional review board. Recruitment procedures, definitions, and confirmation of subject diagnoses are according to previous reports.[11] Genomic DNA of each individual was prepared from leukocytes in EDTA-anticoagulated blood, using PureGene extraction columns (Gentra Biosystems).

Polymorphism Discovery

Because allele frequencies and haplotypes may differ substantially across biogeographic ancestries, a series of 160 individuals (ie, 2n 320 chromosomes; Supplemental Table I) 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 discovery by resequencing. Characterization of 320 chromosomes afforded us the 99% power for discovery of polymorphisms with as low as 1.4% minor allele frequency. Ethnicity was established by self-identification. None of the subjects had a history of secondary hypertension, renal failure, or diabetes mellitus.

Primary Care Population with Extremes of High and Low Blood Pressure

From a database of more than 53 000 people (27 478 females and 25 528 males) in southern California, we ascertained 1182 European-ancestry individuals, of both sexes, from the highest and lowest 5th percentiles of a primary care population[12] in diastolic blood pressure (DBP) distribution. This population sample afforded us 90% power to detect genotype association with a trait when the genotype contributes as little as 3% to the total variation in males; the power is even higher in the females.[13] Evaluation included physical examination, blood chemistries, hemogram, and extensive medical history questionnaire. A total of 1.98% of subjects were excluded because of elevated serum creatinine (1.5 mg/dL).

Twin Pairs

Twins enable estimation of heritability for any trait. In studies of *CHGB* heritability, as well as the influence of *CHGB* polymorphism on *CHGB* expression in vivo and the cold stress test in vivo, 171 twin pairs (342 individuals) were evaluated. Zygosity (69% monozygotic and 31% dizygotic pairs) was confirmed by extensive microsatellite and single-nucleotide polymorphism (SNP) genotyping, as described.[14] Twins ranged from 15 to 84 years; 10% were hypertensive. All of the twins in these allelic/haplotype association studies were self-identified as of European (white) ancestry, to guard against the potentially artifactual effects of population stratification.

Molecular Genetics

Details on resequencing of CHGB locus and genotyping of CHGB variants are available in the Data Supplement.

Phenotyping

Details on biochemical phenotyping and physiological phenotyping: environmental (cold) stress test in twin pairs are available in the Data Supplement.

Statistical Analyses

Estimates are stated as mean value 1 standard error. 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 by using the variance component method implemented in the Sequential Oligogenic Linkage Analysis Routines package www.sfbr.org/solar/ .[15]

Haplotypes were inferred from common SNPs (minor allele frequency 10%) of *CHGB* by using either the HAP program,[16] which can also generate a likely phylogeny for each variant, or by PHASE.[17] Pairwise linkage disequilibrium (LD) between common SNPs was quantified as D by the Graphical Observation of Linkage Disequilibrium software package.[18] Two-way ANOVA or multivariate general linear modeling, using post hoc Bonferroni corrections, was performed in SPSS (Chicago, Ill) to evaluate the significance of single variants and interaction of variants during in vivo association studies as well as in vitro haplotype-specific *CHGB* promoter/reporter activity. Haplotypes in the blood pressure (BP) extreme population were estimated from unphased diploid genotypes by use of the SNP-Expectation Maximization (SNP-EM) program, which includes omnibus likelihood ratio permutation tests.[19] Comparison of haplotype frequencies between population BP extremes (hypertensive cases versus controls) was performed using the SNP-EM algorithm.[20] SNP-EM estimates haplotype frequencies for each group using the EM algorithm, taking into account the probability of all possible haplotype pairs, and calculates an omnibus likelihood ratio statistic to compare haplotype frequencies between 2 groups (cases versus controls), and a permutation test to determine significance in the face of multiple comparisons (set at 10 000 permutations). SNP-EM was used to perform a "sliding window" analysis to identify associated haplotype lengths (from 1 to 4 SNPs) across the locus,[21] thus evaluating all possible haplotypes across the 4 SNPs, thereby interrogating genetic variation at the locus in an unbiased, hypothesis-free way. Additional permutation tests[22] on 3 2 contingency tables (diploid genotype versus BP status), implemented at http://www.physics.csbsju.edu/stats/exact.html, were used to confirm genotype effect on the dichotomous BP trait.

For twin pair analyses, descriptive (genotype-specific mean and standard error) and inferential (^{2}, probability value) statistics were computed across all of the twins, using generalized estimating

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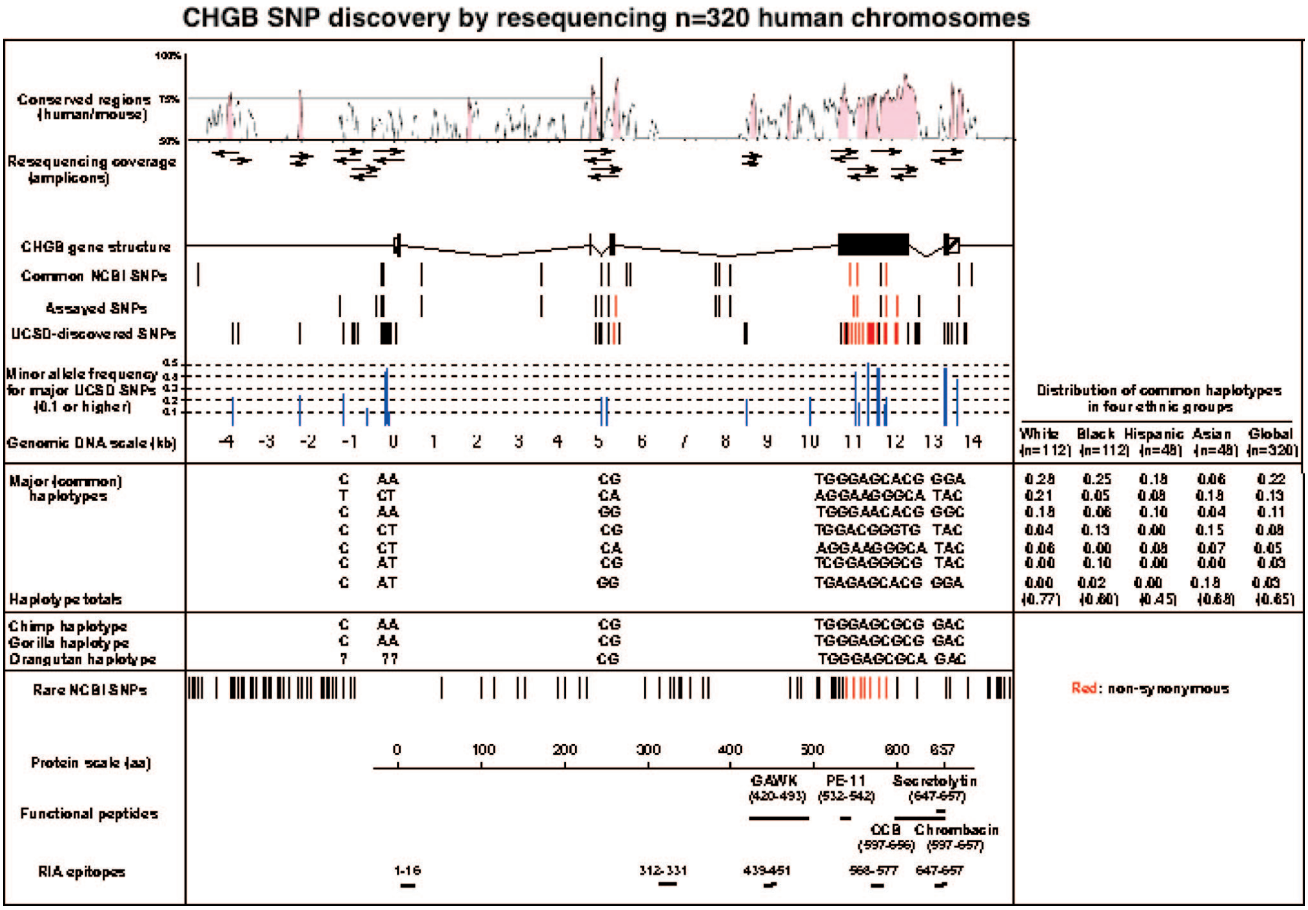

Figure 1. Human *CHGB* genetic diversity. *CHGB* systematic SNP discovery by resequencing all exons, exon/intron borders, UTRs, and proximal promoter (in 320 human chromosomes). Conserved regions between mouse and human *CHGB* were visualized with VISTA.[44] Red rods represent nonsynonymous SNPs, whereas black rods represent synonymous SNPs. Computationally reconstructed haplotypes (by PHASE, using 19 variants across the locus) are indicated, along with their relative frequencies in ethnogeographic groups within our sample populations. Nucleotide deletions in haplotype sequences are indicated by an asterisk. Common NCBI SNPs: SNPs in the public database with reported minor allele frequency 10%. Assayed SNPs: Variants whose assays were attempted in DNAs from individuals phenotyped at UCSD. ? indicates data uncertainty in orangutan upstream sequence.

equations (GEEs; PROC GENMOD) in SAS (Statistical Analysis System; Cary, NC), to account for correlated trait values within each twins, using an exchangeable correlation matrix.[23] Details on CHGB promoter/luciferase reporter activity assays and generation and phenotyping of mouse CHGB-targeted gene ablation (knockout) animals are available in the Data Supplement. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

Results

SNP Discovery

In 160 individuals of diverse biogeographic ancestries, we identified 52 SNPs and 1 single base insertion/deletions in 12 amplicons spanning a 5935 bp footprint, 50% of which were not in the previous public databases (eg, dbSNP) (Supplemental Table II; Figure 1). Of these, 22 SNPs were common (at minor allele frequency 5%); 8 SNPs with exceedingly high minor allele frequencies (40%) were noted in the proximal promoter, exon 4, and intron 4 (Figure 1). There were 14 SNPs in the promoter region, 1 in the 5 -untranslated region (UTR), 21 in the coding exons, 14 in introns, and 3 in the 3 -UTR (Supplemental Table II). Global minor allele frequencies ranged from 0.6% to 48.7%.

When comparing results across ethnicities, some SNPs were common in each of the 4 groups sampled. On the other hand, the frequencies of some SNPs differed substantially across ethnicities: eg, A is the major allele of promoter SNP A-261T in whites, but the minor allele in blacks, Asians, and Hispanics. We also resequenced *CHGB* from 3 nonhuman primates (chimp, gorilla, orangutan) to determine the likely ancestral alleles at polymorphic sites (Figure 1); the most common human haplotype matched the chimp at 15 of 18 sites; at the remaining 3 sites (A11727G, G13383A, and A13612C), the chimp allele was found in less common human haplotypes.

SNP Distribution: Haplotypes

To identify variants that are linked in the population, we inferred haplotypes from diploid genotypes at 18 common (minor allele frequency 5%) SNPs at *CHGB* (Figure 1), stretching 14 kbp from the proximal promoter (C-1239T) to the 3 -UTR (C13612A). Initially using PHASE,[17] we identified 7 major haplotypes spanning the locus (Figure 1), accounting for 65% of chromosomes examined. These 7 haplotypes include common variation in coding and regula-

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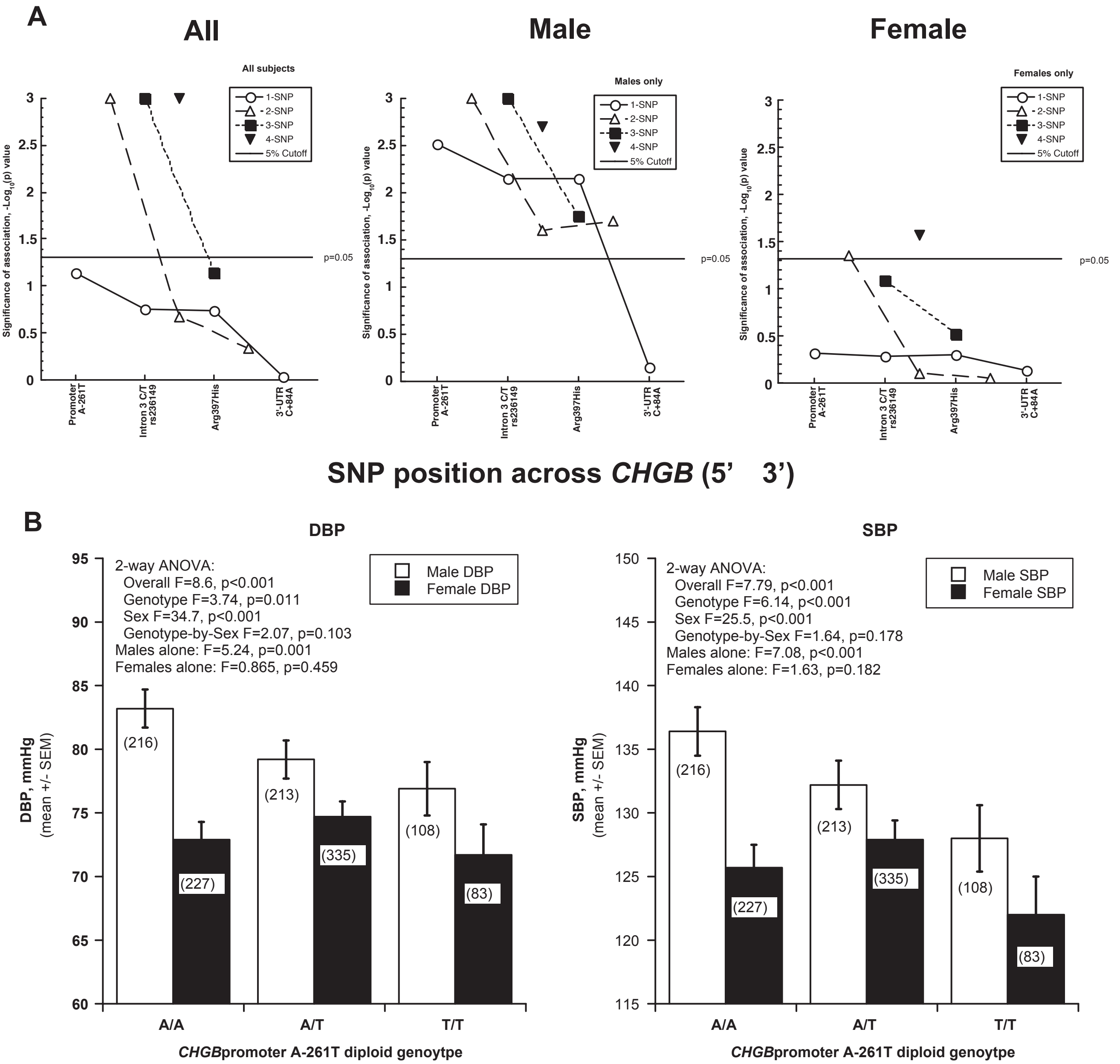

Figure 2. Association the *CHGB* locus with hypertension. A, Haplotype sliding window analysis. Results of significance at each genotyped position or haplotypes are shown as reciprocal probability values, resulting from SNP-EM analyses in the BP extreme populations, for the dichotomous trait of BP status (high vs low). Haplotype analyses were performed on sliding windows of 1, 2, 3, or 4 SNPs at a time. B, *CHGB* promoter polymorphism A-261T: Sex-specific effects on DBP and SBP in the population. The A (major) allele was predictive of higher DBP and SBP in the population as a whole as well as in the males alone, but not the females alone. Results were analyzed by univariate ANOVA.

tory regions and act by LD to span other regions that might influence *CHGB* gene function. The frequencies of these 7 haplotypes varied across ethnicities (Figure 1), with haplotype 1 the most common in whites/blacks/Hispanics, but haplotype 7 the most common in Asians.

LD Across the *CHGB* Locus

We scored 11 variants (Supplemental Table III) spanning the locus in n 468 subjects (2n 936 chromosomes) of European ancestry. To visualize patterns of SNP associations, pair-wise correlations among the 11 common SNPs were quantified as LD parameter D by Graphical Observation of Linkage Disequilibrium[18] across the *CHGB* locus. In these subjects, a single block of LD was maintained across much of the 14 kbp locus (D 0.8) (Supplemental Figure I).

CHGB Genetic Variation in 1182 Subjects with the Most Extreme BP Values in the Population

Given the relatively high degree of LD across the locus, we selected 4 SNPs with high minor allele frequencies (each at 25%), each in Hardy Weinberg equilibrium, to span 4 structural/functional domains across the gene (promoter, intron, exon, 3 -UTR) in this case/control study. Character-

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istics of the 4 SNPs and their major haplotypes are shown in Figure 2 and Supplemental Table IV.

Initially, association of BP status with *CHGB* polymorphism was performed by a haplotype "sliding window" analysis in SNP-EM (Figure 2A). When all subjects (male and female) were included, none of the 4 variants alone was predictive of BP status; but the most 5 2-SNP haplotype (across A-261T, C10501T) associated with BP status (*P* 0.001); haplotypes formed from 3 SNPs (A-261T, C10501T, G11873A; *P* 0.001) or all 4 SNPs (*P* 0.001) were also associated with BP status.

When the SNP-EM analysis was confined to male subjects, single SNPs at A-261T, C10501T, or G11873A were each predictive of BP status (*P* 0.01), with the most significant association by promoter variant A-261T (*P* 0.003; omnibus likelihood ratio statistic 11.36). Haplotypes composed of 2, 3, or 4 contiguous SNPs could were associated with BP status (each at *P* 0.05; omnibus likelihood ratio statistics of 22.6, 29.1, and 41.1, respectively).

When the SNP-EM analysis was confined to female subjects, no single SNP was predictive of BP status by itself (*P* 0.05); when 2 nearby SNPs were included, only the haplotype using the 5 -most SNPs (A-261T, C10501T) was associated with BP status (*P* 0.05); and when 3 nearby SNPs were tested, no haplotype was predictive of BP status. Haplotypes spanning all 4 SNPs were associated with BP status (*P* 0.05).

Because the peak SNP and haplotype effects seemed to occur toward the 5 -end of the gene (Figure 2A), we also analyzed the association of promoter A-261T genotype and BP as a quantitative trait (Figure 2B), revealing effects apparently confined to the male sex. In the promoter region (A-261T), the effect of the A-261 allele is to raise both systolic blood pressure (SBP; *P* 0.001) and DBP (*P* 0.001) in males.

Coding Region

We identified 21 SNPs in the coding region (Supplemental Figure III), among which 15 nonsynonymous SNPs encoded amino acid substitutions, as well as 1 SNP in the 5 -UTR and 3 in the 3 -UTR. Glu247Arg (0.6%) disrupts an acidic domain (Glu$_{241}$-Asp$_{249}$) likely to be important for trafficking of CHGB into secretory granules.[2] Gly266Arg (0.6%) creates a dibasic site (Arg$_{266}$Arg$_{267}$), likely a recognition site for prohormone processing enzymes.[24]

Most of the coding region SNPs lie toward the amino-terminal region of the protein, rather than in the carboxy-terminal region encoding the 5 best-studied peptides, ie, GAWK, PE-11, secretolytin, CCB, or chrombacin, suggesting relative conservation of sequence in this region (Supplemental Figure III).

Heritability and the Effect of *CHGB* Variation on Gene Expression in 171 Twin Pairs In Vivo

The plasma concentrations of 3 CHGB regions assayed seemed to be under substantial genetic control, with h^2 ranging from 50% to 90% (Supplemental Figure IV), and maximal h^2 for CHGB$_{439 \text{ to } 451}$ (at 91 9%, *P* 0.0001), encoded by exon 4 (Supplemental Figure III).

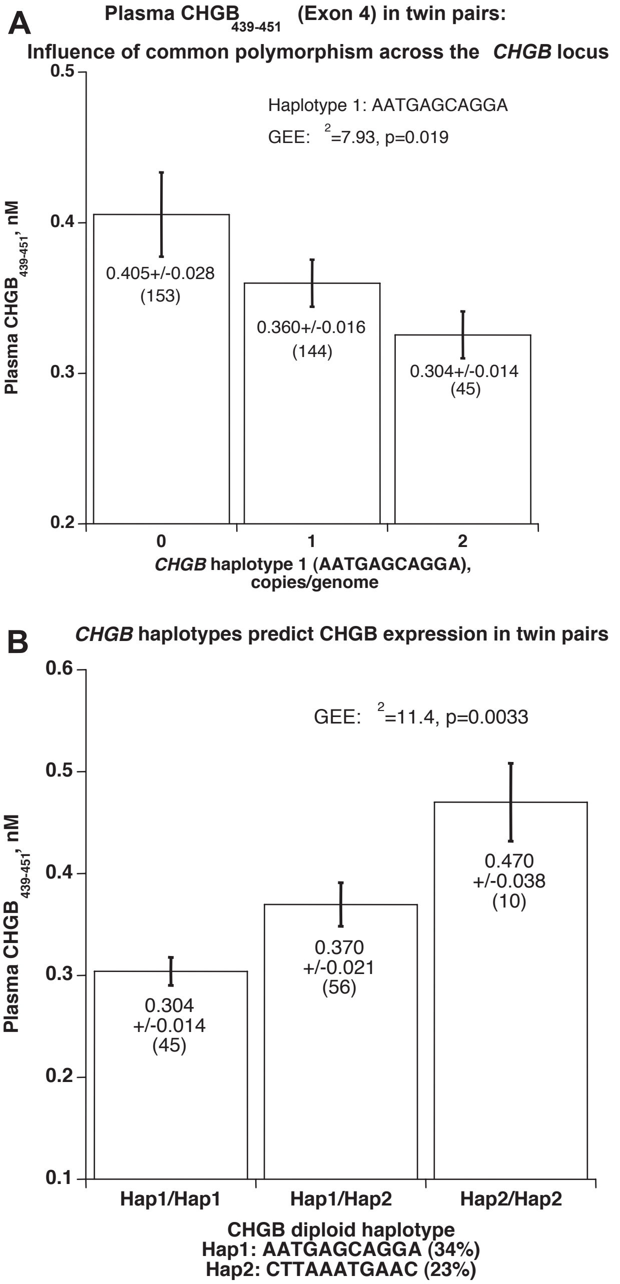

Figure 3. Coding region SNPs and human plasma CHGB. A, *CHGB* haplotypes and SNPs predict CHGB expression in twin pairs. Plasma CHGB$_{439 \text{ to } 451}$ concentrations were measured in twin pairs, and haplotypes were inferred from 11 common SNPs spanning the *CHGB* locus. The 2 most common haplotypes are shown (haplotype 1 at 34% of chromosomes, haplotype 2 at 23%). Increasing haplotype 1 copy number decreases CHGB$_{439 \text{ to } 451}$ expression. Haplotype 1 bears the alleles A-261 3 C10501 3 G11783 3 13612A. B, Diploid haplotypes (diplotypes). The 2 most common haplotypes were expressed as diploid haplotypes, and trait mean SEM values (by GEE) are shown for Hap1/Hap1 homozygotes, Hap1/Hap2 heterozygotes, and Hap2/Hap2 homozygotes. Haplotype 2 bears these alleles 261T, 10501T, 11783A, and C13612.

To probe the functional significance of common variation at *CHGB*, we examined the influence of common polymorphism on the expression of *CHGB* gene products in vivo. Because CHGB$_{439 \text{ to } 451}$ bears the highest heritability in CHGB fragments, we assayed plasma CHGB$_{439 \text{ to } 451}$ in

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n 171 twin pairs (342 individuals) typed for 11 common SNPs spanning the locus (Supplemental Table III); these 11 variants were chosen for very dense spacing across the locus (every 1.3 kbp across 14 kbp), high minor allele frequency (20% for each), and each in Hardy Weinberg equilibrium. We then inferred haplotypes in the twins, using HAP[16] and the 11 SNPs (Supplemental Table V). Copy number of the most common haplotype spanning the locus (haplotype 1, at 34% of chromosomes; AATGAGCAGGA) was predictive of plasma CHGB$_{439\text{ to }451}$ (*P* 0.019, Figure 3A): increasing copy number (03132 copies/genome) progressively decreased the peptide by 33%. When we considered haplotype pairs (diplotypes), combinations of haplotypes 1 and 2 (haplotype 2 is CTTAAATGAAC, at 23% of chromosomes) revealed a progressive increase in plasma CHGB$_{439\text{ to }451}$, by 55% (*P* 0.0033), from haplotype 1 homozygotes through haplotype 2 homozygotes (Figure 3B).

Twin pairs also allowed us to estimate the h^2 of other "intermediate" traits for future development of hypertension,[14] such as urine epinephrine excretion (at h^2 67.6 4.9%, n 316, *P* 0.0001) and the DBP response to cold stress (at h^2 32 8%, n 326, *P* 0.0003).

Sex Influence on *CHGB* Secretion and "Intermediate" Phenotypes for Later Development of Hypertension (in 171 Twin Pairs)

Because sex had a profound effect on the association between *CHGB* genotype and resting BP in the population (Figure 2A and 2B), we used our set of predominantly healthy, normotensive twin pairs and siblings to explore the effect of sex on both CHGB secretion and other early, "intermediate" phenotypes for later development of hypertension[25]: the BP response to environmental (cold) stress (Figure 4A) and epinephrine secretion (Figure 4B). Plasma CHGB$_{439\text{ to }451}$ concentration was lower (*P* 0.015) in males (0.313 0.012 nM, n 203) than females (0.349 0.008 nM, n 544). By contrast, the DBP was higher (*P* 0.014) in males (14.3 1.6 mm Hg, n 62) than females (11.3 0.8 mm Hg, n 236); similarly, urine epinephrine secretion was lower (*P* 0.028) in females (9879 686 ng/g, n 267) than males (13526 1337 ng/g, n 77). Thus women, a group at decreased risk for development of hypertension,[13] displayed not only diminished pressor responses and catecholamine secretion, but also elevated CHGB biosynthesis/secretion.

CHGB Polymorphism: Effects on Biochemical and Physiological Intermediate Traits (in 171 Twin Pairs)

In the twin sample, genetic variation across *CHGB* (Figure 4A), as captured by common haplotype 2 spanning 11 variants (CTTAAATGAAC, at 23%), was associated with not only CHGB secretion but also the BP response to stress. Plasma CHGB$_{439\text{ to }451}$ concentration was lower (*P* 0.0243) in subjects without haplotype 2 (0.335 0.011 nM, n 186) than in those who carried 1 or 2 copies of that haplotype (0.376 0.014 nM, n 142). By contrast, the DBP was *higher* (*P* 0.0073) in haplotype 2 noncarriers (12.4 1.1 mm Hg, n 186) than in carriers (8.9 1.0 mm Hg, n 142). Similarly, increasing *CHGB* haplotype 2 copy number was predictive of decreased catecholamine secretion (Figure 4B). Thus, haplotype 2 appeared to affect CHGB expression and adrenergic/pressor responses inversely, suggesting that subjects with a genetically programmed (ie, *cis*-QTL) increase in CHGB synthesis exhibit greater autonomic stability and thereby decreased cardiovascular risk.

CHGB Promoter Variation: Coordinate Directional Function both In Cella and In Vivo (Twins)

Because common promoter variant A-261T associated with BP (Figure 2A and 2B), we studied the effect of A-261T alleles on transfected *CHGB* promoter strength in chromaffin cells (Figure 4C), using a 1365 bp proximal promoter fragment driving expression of a luciferase reporter in vector pGL3-Basic, and varying the allele at position 261 from T to A by site-directed mutagenesis, followed by sequence verification. Because plasmids carry only 1 genotype (either T or A), we plotted both phenotype values for homozygotes (A/A or T/T) only. Transversion from A to T at 261 resulted in an increase in luciferase expression (*P* 0.0086), which paralleled an increase (*P* 0.035) in plasma CHGB$_{439\text{ to }451}$ expression in vivo in twins selected for 261 homozygosity: 0.333 0.015 nM in A/A homozygotes (n 126) versus 0.372 0.021 nM in T/T homozygotes (n 58). Thus, A-261T variation exerted parallel effects in cella and in vivo.

CHGB Promoter Variation: Reciprocal Effects In Vivo on Gene Expression and Basal BP in the Population

To probe the consequences of CHGB expression in vivo for long-term control of BP (Figure 4C), we plotted plasma CHGB (in twin pairs) versus resting/basal DBP (in the population BP extremes), simplifying the plot by focusing on homozygotes. The same allele (T) that raised CHGB expression also lowered population DBP (from 78.3 1.0 to 74.5 1.6 mm Hg, *P* 0.011).

BP After Targeted Ablation of the *CHGB* Gene in Mice

Chgb / mice displayed substantially higher SBP/DBP (by 20/ 18 mm Hg) than *Chgb* / mice. SBP was 112.0 1.7 mm Hg for *Chgb* / mice (n 12), rising to 131.8 2.3 mm Hg for *Chgb* / mice (n 12, F 47.8, *P* 0.001). DBP was 91.3 1.8 mm Hg for *Chgb* / mice (n 12) and 109.3 2.6 mm Hg for *Chgb* / mice (n 12, F 32.7, *P* 0.001, Figure 5). This finding is consistent with the relationship between plasma *Chgb*$_{439\text{ to }451}$ concentration and BP that we found in humans (Figure 4C).

Discussion

Overview

Patients with hypertension often exhibit increased sympathetic activity,[26,27] and people with sympathetic overactivity tend to develop hypertension.[28,29] Suppression of CHGB expression in neuroendocrine PC12 cells leads to a reduction in the number of catecholamine secretory granules, whereas ectopic expression of CHGB in nonneuroendocrine cells,

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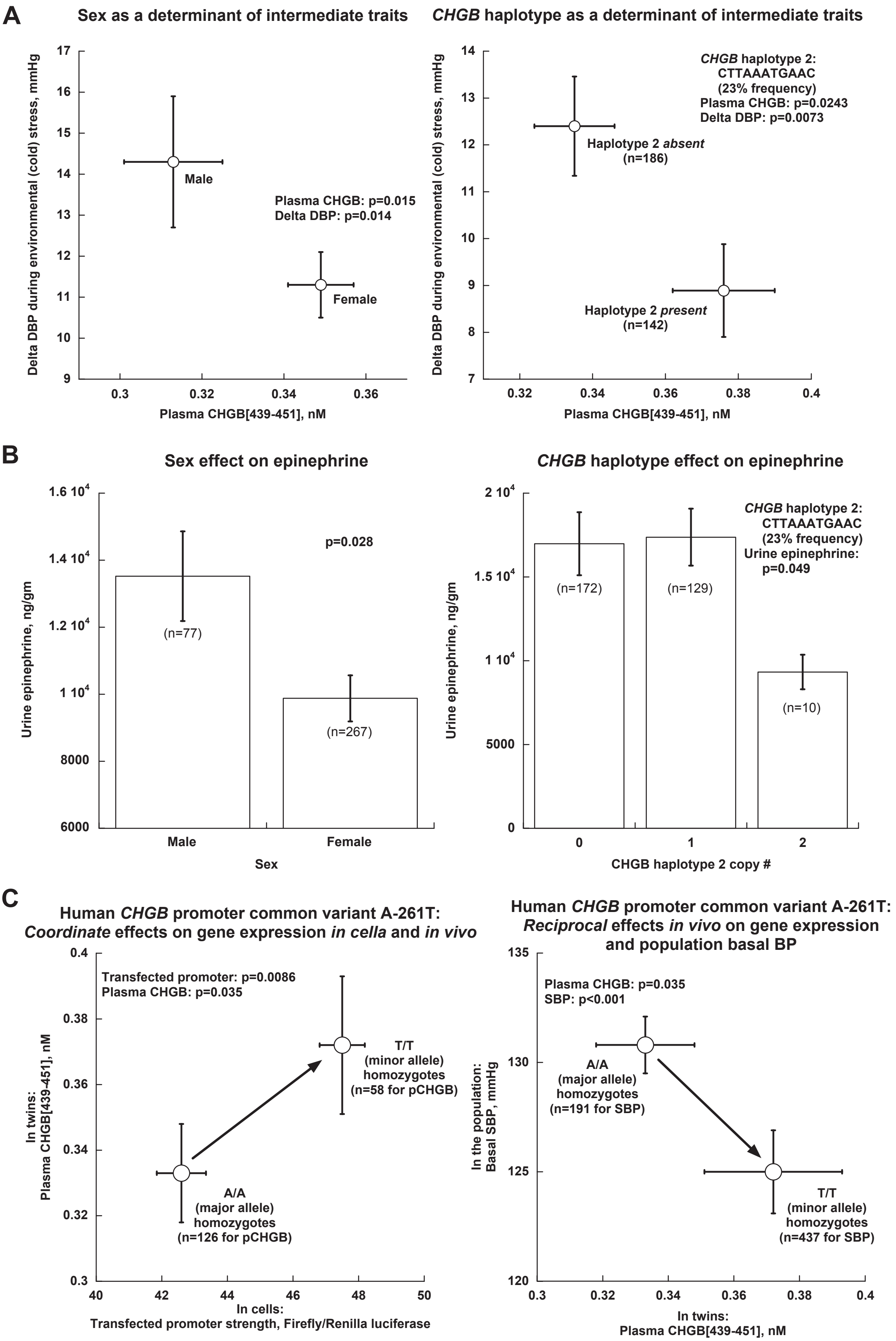

Figure 4. Sex and *CHGB* genetic variation: Pleiotropic effects on biochemical (CHGB$_{439\text{ to }451}$) and physiological "intermediate" phenotypes. A, Sex, *CHGB* genetic variation, and 2 intermediate traits in twin pairs: CHGB secretion and the BP response to environmental stress. *CHGB* haplotype 2 was inferred from 11 common SNPs across the locus. Males have lower CHGB secretion but higher DBP

(*Continued*)

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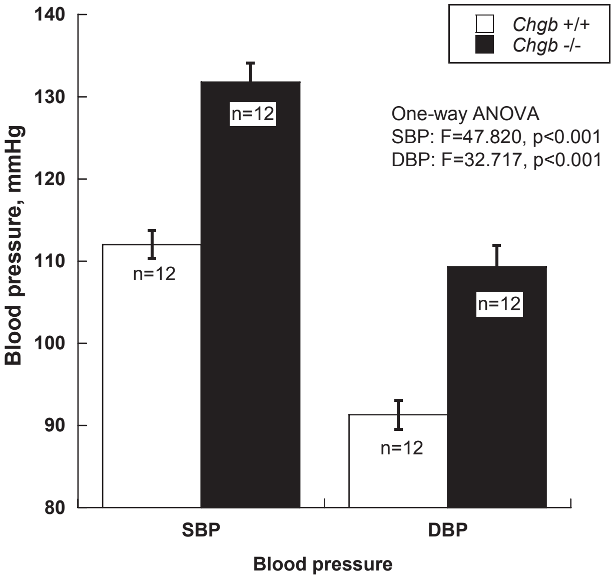

Figure 5. BP in *Chgb* deletion mice. BP was measured over 7 days (3 times/day, or 21 times) in each animal. After *CHGB* deletion, both DBP and SBP increased significantly (P 0.001).

which normally do not contain any secretory machinery, leads to granule biogenesis.[7] In light of the emerging secretory biology of CHGB, we undertook the present study, using the tools of genome technology and statistical genetics to probe how heredity shapes human functional responses in the sympathetic neuroeffector junction, using CHGB as a likely focal point in the pathogenesis of essential hypertension.

Polymorphic Profile at the *CHGB Locus*

Systematic identification of genetic variants at a candidate locus is a strategy for disease association.[30] At *CHGB*, we therefore resequenced all 5 exons, adjacent intronic regions, and the proximal promoter in n 160 human subjects from 4 biogeographic ancestry groups, thereby identifying 53 polymorphisms over a 5935 bp footprint, or just under 1 variant every 100 bp. The genetic diversity that we report at *CHGB* is somewhat more comprehensive than that previously reported for 32 individuals from the Han Chinese population (15 SNPs),[31] or 24 individuals of Japanese ancestry (24 SNPs),[32] as a consequence of the greater number of individuals studied, the multiethnic samples, and the greater resequencing footprint, but no inconsistencies were noted. In the previous Asian reports,[31,32] *CHGB* polymorphisms (mainly toward the 3 end of the gene) were associated with schizophrenia; because the population prevalence of schizophrenia is only 0.4% to 0.6%,[33] our resequenced sample of n 160 normotensive and hypertensive individuals from southern California does not have statistical power to detect such an effect on neuropsychiatric disease. Although the pattern of LD across the *CHGB* locus suggested a extended single block of LD across the 14 kbp locus in individuals of European ancestry (Supplemental Figure I), allele frequencies did differ substantially across the 4 biogeographic ancestries sampled (Supplemental Table II).

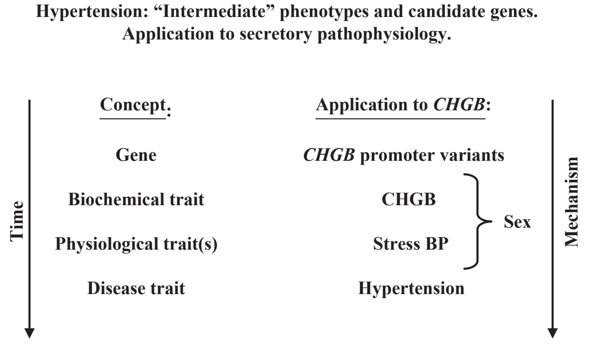

Figure 6. Hypertension: "intermediate" phenotypes and candidate genes. Application to secretory pathophysiology. *CHGB* promoter polymorphisms lead to variation of gene expression, thus changing the secretion of epinephrine, which alters the BP response to environmental stress. After decades, fixed BP elevation (hypertension) may occur in genetically susceptible subjects.

Strategies for Hypertension Risk: Heritability and *CHGB* Variant Effects on Multiple "Intermediate Phenotypes" in Twin Pairs

We developed series of twin pairs of southern California, typed for traits likely to contribute to later development of hypertension.[11] The twin data offer the advantage of determining trait heritability (h^2), the fraction of phenotypic variance accounted for by genetic variance, a logical estimator of the tractability of any trait to genetic investigation; indeed, the twin traits were substantially heritable (Supplemental Figure IV).

Multiple autonomic phenotypes in the twins, both biochemical and physiological, allowed construction of an integrated picture of the effects of genetic variation at *CHGB* on a very proximate biochemical phenotype, plasma CHGB concentration (Figure 4A); a later biochemical consequence, epinephrine excretion (Figure 4B); a more distant physiological consequence, change in BP during environmental stress (Figure 4A); and finally basal/resting BP in the population (Figure 2B).

Intriguingly, the same *CHGB* genetic variants that increased CHGB storage and secretion (Figure 4A) also decreased catecholamine secretion (Figure 4B), the sensitivity of BP to environmental stress (Figure 4A), and finally basal BP in the population (Figure 2B). A unifying hypothesis is presented in Figure 6. Such a hypothetical framework is supported by the experimental findings of Huh et al,[7] who found by overexpression and underexpression of CHGB in catecholaminergic cells that CHGB regulates the biogenesis

Figure 4 Continued. response to cold stress (both P 0.02), whereas the presence of haplotype 2 (1 or 2 copies) is predictive of both higher CHGB secretion and lower DBP response (both P 0.03). B, Sex, *CHGB* genetic variation, and catecholamine secretion in twin pairs. *CHGB* haplotype 2 was inferred from 11 common SNPs across the locus. Males have higher urine epinephrine, whereas haplotype 2 homozygosity is predictive of lower urine epinephrine excretion (both P 0.05). C, *CHGB* promoter variant A-261T: phenotypic pleiotropy. T/T (minor allele) homozygosity increases gene expression in cells, increases plasma CHGB secretion in twins, but decreases BP in the population.

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of hormone storage granules of the regulated secretory pathway. Absence of CHGB would therefore be predicted to disrupt the pathway, perhaps leading to constitutive (unregulated or autonomous) transmitter release. Indeed, we observed excess catecholamine secretion (Figure 4B) and autonomic BP instability (Figure 4A) in subjects with genetically programmed decrease in CHGB biosynthesis and secretion (Figure 4A). Evidence in cella[34] and in vivo[35] also supports a critical role for the CHGB paralog CHGA in the biogenesis of catecholamine storage vesicles; indeed, targeted ablation of CHGA[35] results in dysregulated catecholamine storage and secretion, accompanied by systemic hypertension.

In this report, we focused primarily on 2 indices of sympathoadrenal function: catecholamine secretion (Figure 4B) and the BP response to environmental stress (Figure 4A). Because CHGB has such a widespread occurrence in amine and peptide storage vesicles,[2,3,9,10,36,37] it is likely that additional consequences of *CHGB* genetic variation might be uncovered in other branches (eg, parasympathetic) of the autonomic system, or the wider neuroendocrine system. However, such alterations are beyond the scope of this initial report.

Established Hypertension and *CHGB* Genetic Variation: Population BP Extremes

To pursue the genetic involvement in established essential hypertension, we developed a powerful resource in a sample set consisting of individuals with extremely high and low BPs. From a population sample of more than 53 000 people, we ascertained 1100 age-, gender-, and ethnicity-matched individuals from the upper and lower 5th percentiles of the population BP distribution.[13]

Using *CHGB* polymorphisms spanning the locus (Supplemental Figure II), we took a haplotype “sliding window” approach in SNP-EM[19] to test the effects of CHGB regions on the hypertension trait. We found that variation across the locus was predictive of BP category (Figure 2A), and the SBP and DBP quantitative traits (haplotypes, Figure 2B), but the effects were substantially more impressive in males than females, and the genetic effects in males seemed to peak toward the 5 (promoter) end of the gene, with the most significant single effect at promoter variant A-261T.

When we studied the effect of A-261T variation on the SBP/DBP quantitative traits (Figure 2B), once again the effects were most impressive for males (P 0.001/P 0.001) than females (P 0.182, P 0.459). The profound difference in genetic effects between sexes led us to explore the effects of sex at earlier stages in the hypothetical phenotypic chain between gene and the ultimate disease trait (Figure 6; see below). Of note, sex itself had a profound effect on BP in the population (Figure 2B), consistent with epidemiological findings over several decades.[38]

Although we undertook *CHGB* polymorphism discovery systematically across several biogeographic ancestry groups (Supplemental Tables I and II; Figure 1), we conducted *CHGB* marker-on-trait BP studies in subjects of a single ancestry: European (Figure 2A and 2B). We restricted our initial analyses to subjects of 1 ancestry because allelic association studies can be susceptible to artifactual conclusions resulting from even inapparent population admixture.[39] Studies of additional ethnic or population groups will be required to evaluate whether our CHGB results are of more general importance in the overall population.

Sex and Genetic Risk of Hypertension: “Intermediate” and Ultimate Disease Traits

Sex exerted profound effects on not only the BP trait in general (Figure 2B)[38] but also the BP response to *CHGB* genetic variation (Figure 2A and 2B). Previously we have noted significant gene-by-sex interactions on BP in the population, and sex differences in the response to adrenergic drug provocations.[40,41] To understand how sex might influence the genetic predisposition to hypertension, we studied the effect of sex on each of the “intermediate phenotypes” influenced by *CHGB* genetic variation. We found that sex systematically influenced each such trait, in ways predicted to reduce risk of future development of hypertension: females exhibited increased CHGB expression (Figure 4A), reduced epinephrine secretion (Figure 4B), and reduced pressor responses to environmental stress (Figure 4A). We noted effects of *CHGB* genetic variation on BP in males though not females (Figure 2A and 2B), and observed that autonomic traits, both biochemical (Figure 4A) and physiological (Figure 4A and 4B), differed in males and females; nonetheless, we did not statistically document significant *CHGB* gene-by-sex interaction on BP (Figure 2B), suggesting caution during interpretation of sex-specific roles of genes in cardiovascular trait determination.

Although we measured BP before and after environmental stress (Figure 4A), we measured catecholamines only before stress. However, previous longitudinal studies indicate that the pressor (BP) response to cold is an effective predictor of future development of hypertension,[42] although the predictive value of such early stress tests seems to be more apparent in males.[43] Thus, at every stage of the putative pathogenic chain (Figure 6) between *CHGB* genetic variation and the development of hypertension, sex may be involved as a potential modifier of gene effects.

Role of the *CHGB* Promoter: Coordinate *CHGB* Genetic Effects In Cella and In Vivo

Because BP in the population was best associated with genetic variation in the *CHGB* promoter region (Figure 2A and 2B), we ligated a 1.4 kb proximal promoter fragment to a luciferase reporter and tested the effect of the A-261T variant on promoter activity in transfected chromaffin cells (Figure 4C). We found that the minor (T) was significantly more active than the A allele in programming transcription; of further note was the coordinate effect of the 2 alleles in cella and in vivo: the T allele not only increased transcription in chromaffin cells, but also increased plasma CHGB in twin pairs. These results are consistent with genetic variation in the *CHGB* promoter initiating the entire cascade of phenotypic events illustrated in our hypothetical schema (Figure 6). Loss-of-function *CHGB* promoter variants (such as allele A at A-261T) would give rise to decreased CHGB expression, thereby unleashing sympathochromaffin activity, leading to

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exaggerated pressor responses to environmental stressors, and ultimately fixed systemic hypertension.

BP in Mice with Targeted Ablation of the *CHGB* Locus

Because we found inverse effects of CHGB expression on catecholamine secretion (Figure 4B) pressor responses (Figure 4A), and because the *CHGB* genotype associated with lower CHGB expression in cella (Figure 4C) and in vivo (Figure 4C) was also predictive of higher resting SBP/DBP in the population (Figure 4C), we hypothesized that experimental disruption of CHGB expression would elevate BP. We tested this hypothesis by targeted ablation of the *CHGB* locus, and as predicted found substantial elevations in both SBP (by 20 mm Hg) and DBP (18 mm Hg) in *Chgb*(/) mice. Thus, experimental evidence further strengthens our clinical conclusions (Figure 6) that *CHGB* genetic variation initiates a cascade of events ultimately resulting in BP disturbances in the population.

Conclusions and Perspectives

Common genetic variation at the *CHGB* locus, especially in the proximal promoter, influences CHGB expression as well as catecholamine secretion in vivo, and later the early heritable responses to environmental stress, and finally resting/basal BP in the population (Figure 6 hypothesis). These changes are modified at each pathophysiologic level by the influence of sex. Although causal inferences in this proposed chain of events are not yet established, the pathway illustrated in Figure 6 does yield testable predictions for experimental verification. These results point to new molecular strategies for probing autonomic control of circulation and, ultimately, the susceptibility to and pathogenesis of cardiovascular disease states such as hypertension.

Sources of Funding

This work was supported by the Department of Veterans Affairs, National Institutes of Health.

Disclosures

None.

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CLINICAL PERSPECTIVE

Hypertension is a complex trait often with deranged autonomic control of the circulation. Chromogranin B (*CHGB*) is the most abundant core protein in human catecholamine secretory vesicles. Here we studied inter-individual variability at the *CHGB* locus and found an association between the 5'/promoter region of *CHGB* and hypertension, most prominently in men. The peak effect was located in the proximal promoter at variant A-261T. The promoter allele that predicted *higher* DBP and SBP also predicted *lower* circulating/plasma CHGB concentration in twin pairs. In twins, the same *CHGB* variants that predicted *lower* basal CHGB secretion also predicted *exaggerated* catecholamine secretion and BP response to environmental (cold) stress. The effect of A-261T on CHGB expression was confirmed in chromaffin cells by site-directed mutagenesis on transfected *CHGB* promoter/luciferase reporter activity. Chgb knockout mice displayed substantially *increased* BP, confirming the mechanistic basis of our findings in humans. We conclude that common genetic variation at the *CHGB* locus, especially in the proximal promoter, influences *CHGB* expression. Ultimately, this leads to catecholamine secretion and the early heritable responses to environmental stress, with consequent changes in resting/basal BP in the population. Both the early (gene expression) and late (population BP) consequences of *CHGB* variation are sex-dependent. The results point to new molecular strategies for probing autonomic control of the circulation, and ultimately susceptibility to cardiovascular disease states such as hypertension and their mechanisms.

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SUPPLEMENTAL MATERIAL.

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Supplementary: Molecular genetics (online supplement).

Resequencing of CHGB locus. Public draft human [12] and mouse[13] 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. The NCBI source for *CHGB* source clones were NM_001819 and NT_011387. Positions were numbered with respect to the mRNA cap (transcriptional initiation) site. PCR primers we designed by Primer3 [14] to span each of the 5 exons, as well as include 50-100 bp of flanking intronic sequence, and 1838 bp of proximal promoter (upstream of the cap site). The PCR, purification, sequencing and analysis of target sequences were according to the regular protocol described previously[15]. Rare SNPs were confirmed by re-sequencing in multiple individuals, or from the reverse direction.

Genotyping of *CHGB* variants. SNP diploid genotypes at *CHGB* were scored by either of two base-extension systems: the MALDI-TOF system of Sequenom [16] or the luminescent system of Pyrosequencing [17]. In each case, initial PCR amplification of the template was followed by primer-mediated base extension across the variant position.

Supplementary: Biochemical phenotyping. EDTA-anticoagulated plasma was obtained from each subject, and stored at -70°C prior to assay. CHGB region-specific radioimmunoassays (for $\mathrm{CHGB}_{312\text{-}331}$, $\mathrm{CHGB}_{439\text{-}451}$, $\mathrm{CHGB}_{566\text{-}577}$) were based on synthetic peptides, as previously described [18, 19]. [^{125}I]-radiolabeling of each peptide was enabled by either an endogenous or adventitious (terminal) Tyr residue. Polyclonal rabbit antisera were developed to the synthetic CHGB regions as described [20]. Catecholamines in urine were determined by radiochemical assay, as previously described [21], and normalized to creatinine concentration.

Supplementary: Physiological phenotyping: Environmental (cold) stress test in twin pairs. To probe the functional significance of common variation at *CHGB*, we examined the potential influence of 11 common CHGB polymorphisms on the blood pressure response during the environmental (cold) stress test [11]of 171 twin pairs (342 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.

Supplementary: *CHGB* promoter/luciferase reporter activity assays. Haplotype-specific promoter fragments corresponding to *CHGB* –1365/+141 bp were PCR-amplified from genomic DNA of known homozygotes, and cloned into promoter-less firefly luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI). PC12 pheochromocytoma cells were transfected (at 50-60% confluence) with 1 μg of supercoiled promoter haplotype-firefly luciferase reporter plasmid and 10 ng of the Renilla luciferase

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expression plasmid pRL-CMV (Promega Inc., Madison, WI) as internal control per well, by the liposome method (Superfect, Qiagen, Valencia, CA). The firefly and renilla luciferase activities in the cell lysates were measured 16-24 hours after transfection, using the Dual Luciferase® reporter assay system (Promega, Madison, WI) and the results were expressed as the ratio of firefly/Renilla luciferase activity as described previously [10, 29]. Each experiment was repeated a minimum of three times. Results were expressed as mean ± SEM. Statistical significance ($p<0.05$) was calculated using the student's or t-test or one-way ANOVA.

Supplementary: Generation and phenotyping of mouse *Chgb* targeted gene ablation (knockout) animals. The targeting construct used for homologous *Chgb* recombination in E14.1-129/Ola ES (embryonic stem) cells included the *H. simplex* virus thymidine kinase gene, 2.8 kbp of mouse *Chgb* proximal promoter region, the neomycin resistance gene, and a 3.6 kbp BglII-EcoRI fragment extending from exon 1 through intron B. ES were cultured in presence of 2 mM ganciclovir (Syntex Pharmaceuticals) and 300 mg/ml neomycin (Gibco). After double selection, clones were screened by Southern blot analysis of HindIII-digested genomic DNA using a 1.6 kb fragment from intron 1 of mouse *Chgb* as probe. Five positive ES cell clones were injected into C57/BL-6 blastocysts, and four generated chimeras showing germline transmission. Subsequent generations were obtained by intercrossing and outcrossing with C57BL/6J mice and genotyped by Southern blot analysis. Finally, lines of *Chgb* wild-type (+/+) and knockout (-/-) mice, on >95% C57BL/6J genetic background, were established from 12 *Chgb*(+/-) founders. Details of the *Chgb* gene-targeted mouse strain have been submitted for publication (W.B. Huttner et al, 2008).

Non-invasive mouse tail-cuff blood pressures were obtained as previously described [30]with a BP-2000 system (Visitech Systems Inc., Cary, NC) on male mice previously acclimated to the instrument 3-4 times daily for 3-5 days.

Supplementary Figure 1: Patterns of linkage disequilibrium across the human *CHGB* locus, based on comprehensive initial resequencing data. SNPs with minor allele frequency >5% were evaluated for pairwise linkage disequilibrium (LD) across the locus, with the GOLD software algorithm. 11 SNPs with minor allele frequency >20% were evaluated for pairwise linkage disequilibrium (LD) across the locus in European ancestral subjects (2n=936 chromosomes). LD is displayed as D' value on a pseudocolor scale: dark blue (D'=0) to bright red (D'=1).

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Supplementary Figure 2: *CHGB* in hypertension: the 4 common variants scored across the locus in population BP extremes. The positions of variants are numbered upstream (-) or downstream (+) of the cap site. Amino acid positions are numbered in the mature CHGB protein, after excision of the 20 amino acid signal peptide. Each SNP is given as major allele/minor allele. The minor allele frequency for each polymorphism is >25%. Red rods represent non-synonymous SNPs, while black rods represent synonymous SNPs.

Supplementary Figure 3: Coding region SNPs, functional domains and epitopes of human CHGB. Amino acid positions are numbered within the mature CHGB protein, after excision of the 20 amino acid signal peptide. Radioimmunoassay (RIA) epitopes: Synthetic peptides upon which CHGB RIAs have been based. Peptides characterized: Peptides previously characterized for biological activity or proteolytic excision from CHGB. Multibasic sites: 2 or more consecutive Lys or Arg residues; putative cleavage sites for prohormone processing proteases. Acidic domains: Regions of 3 or more consecutive Asp or Glu residues.

Supplementary Figure 4: Heritability (h^2) of plasma CHGB concentration by region-specific radioimmunoassays: Studies in twin pairs. The plasma concentrations of three CHGB regions assayed seemed to be under substantial genetic control, with h^2 ranging from ~50-90%. $h^2=V_G/V_P$, where V_G is genetic variance and V_P is total phenotypic variance. Bars give the estimate for each h^2 value, ± one SEM, with significance as p value.

Supplementary Table 1: Systematic polymorphism discovery at the *CHGB* locus in n=160 human subjects (2n=320 chromosomes). Characteristics of resequenced subjects in the study. Values are shown as mean±SEM.

Variables	White (n=56)	Black (n=56)	Hispanic (n=24)	Asian (n=24)	Global (n=160)
Gender (M/F)	40/16	38/18	21/3	13/11	112/48
Age (years)	48±1.5	43±1.6	48±2.0	33±2.9	44±1.0
Hypertension (Y/N)	11/45	10/46	5/19	6/18	32/128
Height (m)	1.74±0.01	1.73±0.01	1.69±0.02	1.66±0.02	1.67±0.02
Weight (kg)	82±2.8	84±3.2	89±4.9	67±2.8	81±1.8
Body surface area (m^2)	1.98±0.03	2.02±0.03	2.04±0.05	1.76±0.04	1.97±0.02
Body mass index (kg/m^2)	27.2±0.9	29.0±1.0	30.7±1.4	24.0±0.9	27.8±0.5
Systolic BP (mmHg)	125±2.3	130±2.6	131±5.0	129±5.5	129±1.6
Diastolic BP (mmHg)	72±1.4	75±1.5	73±2.9	70±2.4	72±0.9

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Supplementary Table 2: Summary of *CHGB* SNP discovery. 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. Variants are presented as major allele/minor allele. Amino acid positions are numbered in the mature CHGB protein, after excision of the 20 amino acid signal peptide. Nucleotide deletion is indicated by *.

						Minor allele frequency				
SNP#	SNP	RefSNP #	Location	Amino acid Change	Position to Cap site	White (2n=112)	Black (2n=112)	Hispanic (2n=48)	Asian (2n=48)	Global (2n=320)
1	T/C	new	Promoter		-3863	0.235	0.172	0.25	0.143	0.205
2	T/C	new	Promoter		-3763	0	0.241	0	0	0.09
3	G/A	rs7260718	Promoter		-2224	0.088	0.362	0	0.5	0.222
4	C/T	rs236139	Promoter		-1239	0.364	0.148	0.304	0.283	0.268
5	T/C	new	Promoter		-1207	0	0.033	0	0	0.013
6	T/C	new	Promoter		-973	0	0.019	0	0	0.006
7	C/A	new	Promoter		-947	0	0.017	0	0	0.006
8	A/G	new	Promoter		-847	0	0.017	0	0	0.006
9	G/A	new	Promoter		-613	0	0.038	0	0	0.013
10	A/C	rs236140	Promoter		-296	0.42	0.317	0.568	0.658	0.444
11	A/T	rs236141	Promoter		-261	0.422	0.519	0.609	0.833	0.456
12	C/T	new	Promoter		-200	0	0.028	0	0	0.01
13	C/G	new	Promoter		-91	0	0	0.063	0	0.007
14	C/T	new	Promoter		-69	0	0.019	0	0	0.006
15	T/C	rs16991480	5'-UTR		55	0	0	0.021	0.229	0.038
16	A/G	new	Intron 2		4873	0	0.019	0.021	0.229	0.045
17	C/T	new	Intron 2		4943	0	0	0.063	0	0.006
18	A/G	new	Intron 2		5017	0	0	0.031	0	0.006
19	C/G	rs236145	Intron 2		5019	0.222	0.16	0.167	0.417	0.223
20	G/A	rs236146	Intron 2		5195	0.324	0.09	0.217	0.271	0.222
21	G/A	new	Exon 3	Ala24Thr	5338	0.023	0	0	0	0.006
22	C/T	new	Intron 3		5451	0	0	0	0.031	0.006
23	*/C	rs11481695	Intron 3		8502	0.191	0.224	0.143	0.25	0.205
24	C/T	new	Intron 3		8503	0	0.034	0	0	0.013
25	T/C	rs6139872	Intron 3		10803	0.009	0.009	0.023	0.152	0.032
26	T/A	rs6085324	Exon 4	Ser73Thr	10900	0.327	0.083	0.239	0.283	0.223
27	G/A	new	Exon 4	Ser73Ser	10902	0.018	0	0	0	0.006
28	G/C	rs236150	Exon 4	Lys97Asn	10974	0.009	0.185	0	0	0.068
29	G/A	new	Exon 4	Ala114Ala	11025	0.018	0.028	0.065	0.022	0.029
30	G/A	rs6133278	Exon 4	Asp125Asn	11056	0.009	0.037	0.043	0.37	0.077
31	G/A	rs910122	Exon 4	Arg158Gln	11156	0.4	0.368	0.477	0.457	0.415
32	A/C	rs881118	Exon 4	Asn180His	11221	0.064	0.25	0.174	0.152	0.158
33	G/A	rs6139873	Exon 4	Arg212Gln	11318	0.009	0.009	0.021	0.13	0.029
34	G/A	rs236151	Exon 4	Thr223Ala	11350	0.213	0.091	0.125	0.043	0.131
35	G/A	new	Exon 4	Arg238Gln	11396	0	0.036	0.021	0.043	0.022
36	A/G	new	Exon 4	Glu247Gly	11423	0	0.02	0	0	0.006
37	G/A	new	Exon 4	Gly266Arg	11479	0	0	0	0.031	0.006
38	C/G	rs236152	Exon 4	Ala333Gly	11681	0.396	0.574	0.5	0.435	0.484
39	A/G	rs236153	Exon 4	Glu348Glu	11727	0.396	0.574	0.5	0.435	0.484
40	C/T	rs742710	Exon 4	Pro393Leu	11861	0.055	0.264	0.217	0.146	0.166
41	G/A	rs742711	Exon 4	Arg397His	11873	0.327	0.1	0.196	0.271	0.22
42	G/A	new	Exon 4	Arg480Lys	12122	0.009	0.009	0.022	0.146	0.032

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43	A/G	new	Exon 4	Lys484Glu	12133	0	0.019	0	0	0.006
44	C/T	new	Exon 4	Leu569Leu	12390	0	0	0.031	0	0.006
45	T/C	rs6117000	Intron 4		12588	0	0.019	0	0	0.006
46	C/T	new	Intron 4		12659	0	0.019	0	0	0.006
47	G/A	new	Intron 4		12662	0.015	0	0	0	0.007
48	G/T	rs236154	Intron 4		13305	0.418	0.433	0.458	0.417	0.487
49	G/A	rs236155	Intron 4		13383	0.409	0.433	0.5	0.438	0.481
50	T/C	new	Exon 5	Leu639Leu	13469	0	0.019	0	0	0.006
51	C/A	rs2821	3'-UTR		13612	0.382	0.346	0.375	0.479	0.39
52	G/A	new	3'-UTR		13774	0.024	0	0	0	0.006
53	A/T	rs236156	3'-UTR		13805	0.009	0.096	0	0	0.035

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Supplementary Table 3: Eleven *CHGB* SNPs genotyped in extensively phenotyped twin pairs. 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.

Location	**Position**	**Allele**		**Amino acid change**	**NCBI RefSNP**	**MAF**	**HWE**	
		Major	**Minor**				χ^2	**p value**
Promoter	-296	A	C	-	rs236140	0.40	1.485521	0.475799
Promoter	-261	A	T	-	rs236141	0.399	1.196656	0.54973
Intron-1	3565	T	G	-	rs236142	0.222	1.0925	0.579117
Intron-2	5195	G	A	-	rs236146	0.243	2.052712	0.35831
Intron-3	7755	A	G	-	rs387700	0.23	0.673258	0.714174
Intron-3	8122	G	A	-	rs454328	0.373	3.464499	0.176886
Intron-3	10501	C	T		rs236149	0.309	1.85827	0.394895
Exon-4	11727	A	G	Asp348Glu	rs236153	0.377	3.996326	0.135584
Exon-4	11873	G	A	Arg397His	rs742711	0.266	2.65808	0.264731
Intron-4	13383	A	G	-	rs236155	0.377	2.555647	0.278643
3'-UTR	13612	C	A	-	rs2821	0.395	4.088273	0.129492

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Supplementary Table 4: CHGB genetic variants scored in population BP extremes. Four common (minor allele frequency >25%) *CHGB* SNPs spanned the locus, and major haplotypes were generated. SNP position is numbered upstream (-) or downstream (+) of the cap site. NCBI RefSNP is the reference number in the NCBI SNP database. MAF: Minor allele frequency. HWE: Hardy-Weinberg Equilibrium.

4A: Individual SNPs spanning the locus.

Position (to cap)		**Alleles**		**Amino acid change**	**NCBI RefSNP**	**MAF**	**HWE**	
		Major	**Minor**				χ^2	**p**
Promoter	-261	A	T	-	rs236141	0.397	0.645	0.724
Intron-3	+10501	C	T	-	rs236149	0.351	1.491	0.474
Exon-4	+11873	G	A	Arg397His	rs742711	0.264	0.028	0.986
3'-UTR	+13612	C	A	-	rs2821	0.387	1.562	0.458

4B: Haplotypes across the *CHGB* locus.

Haplotypes	**Allele sequence (5’→3’)**	**Frequency**
Hap-1	ACGA	0.328
Hap-2	ACGC	0.23
Hap-3	TTAC	0.22

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Supplementary Table 5: *CHGB* haplotype distribution in twins. The 11 variants used in constructing these haplotypes (by the HAP algorithm) were (5'→3'): A-296C, A-261T, G3565T, G5195A, G7755A, G8122A, C10501T, Asp348Glu, Arg397His, A13383G, C13612A.

Haplotype #	Sequence	N (chromosomes)	% of chromosomes
1	AATGAGCAGGA	234	34.2
2	CTTAAATGAAC	158	23.1
3	AAGGGGCAGGC	144	21.1
4	CTTGAATGGAC	61	8.9
5	CTTGAGCAGGA	33	4.8
6	AATGAATGAAC	16	2.3
7	AATGAGCAGGC	8	1.2
8	CTTGAACGGAC	7	1
9	CATGAGCAGGA	4	0.6
10	CTTAAACGAAC	4	0.6
11	CTTAAATGGAC	4	0.6
12	CTTGAATGAAC	2	0.3
13	AATGAGCAGAA	1	0.1
14	AATGAGTAGGA	1	0.1
15	AATAAATGAAC	1	0.1
16	AAGGGGCAGGA	1	0.1
17	ATTGAATGGAC	1	0.1
18	ATTAAATAGGA	1	0.1
19	ATTAAATGAAC	1	0.1
20	CTGGGGCAGGC	1	0.1
21	CTGGGGTAGAC	1	0.1

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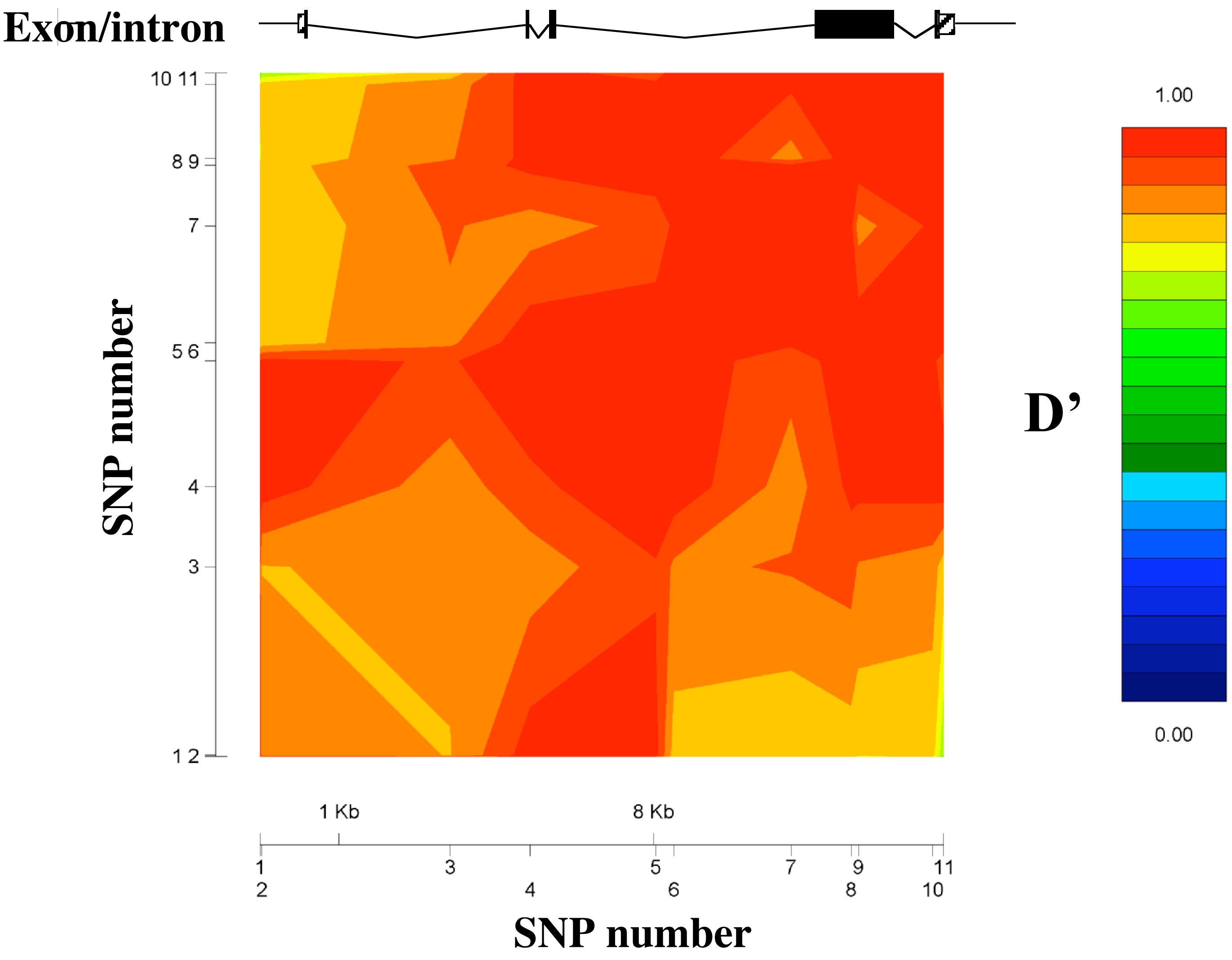
CHGB: Graphical Overview of Linkage Disequilibrium in white subjects (n=936 chromosomes)



On-line supplementary Figure 1

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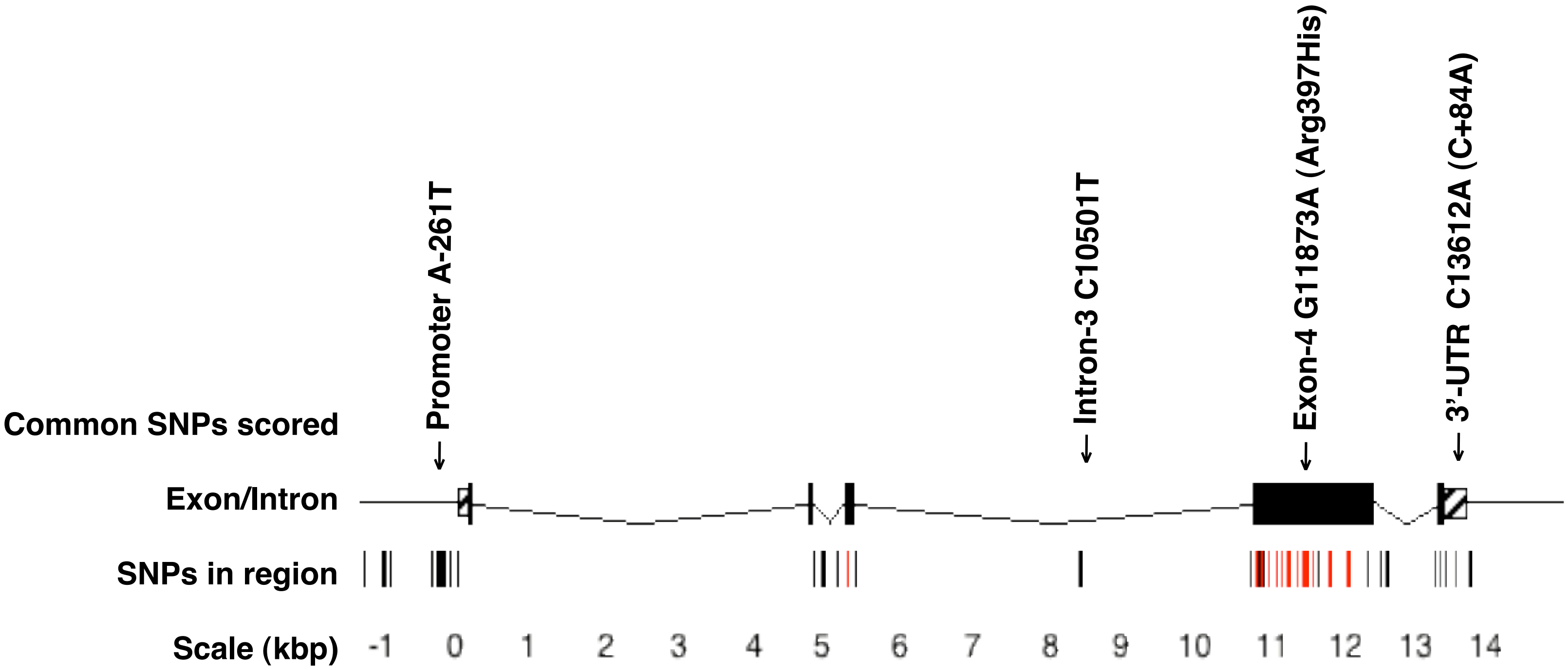
Chromogranin B (*CHGB*) in hypertension:

4 common variants scored across the locus in population BP extremes



On-line supplementary Figure 2

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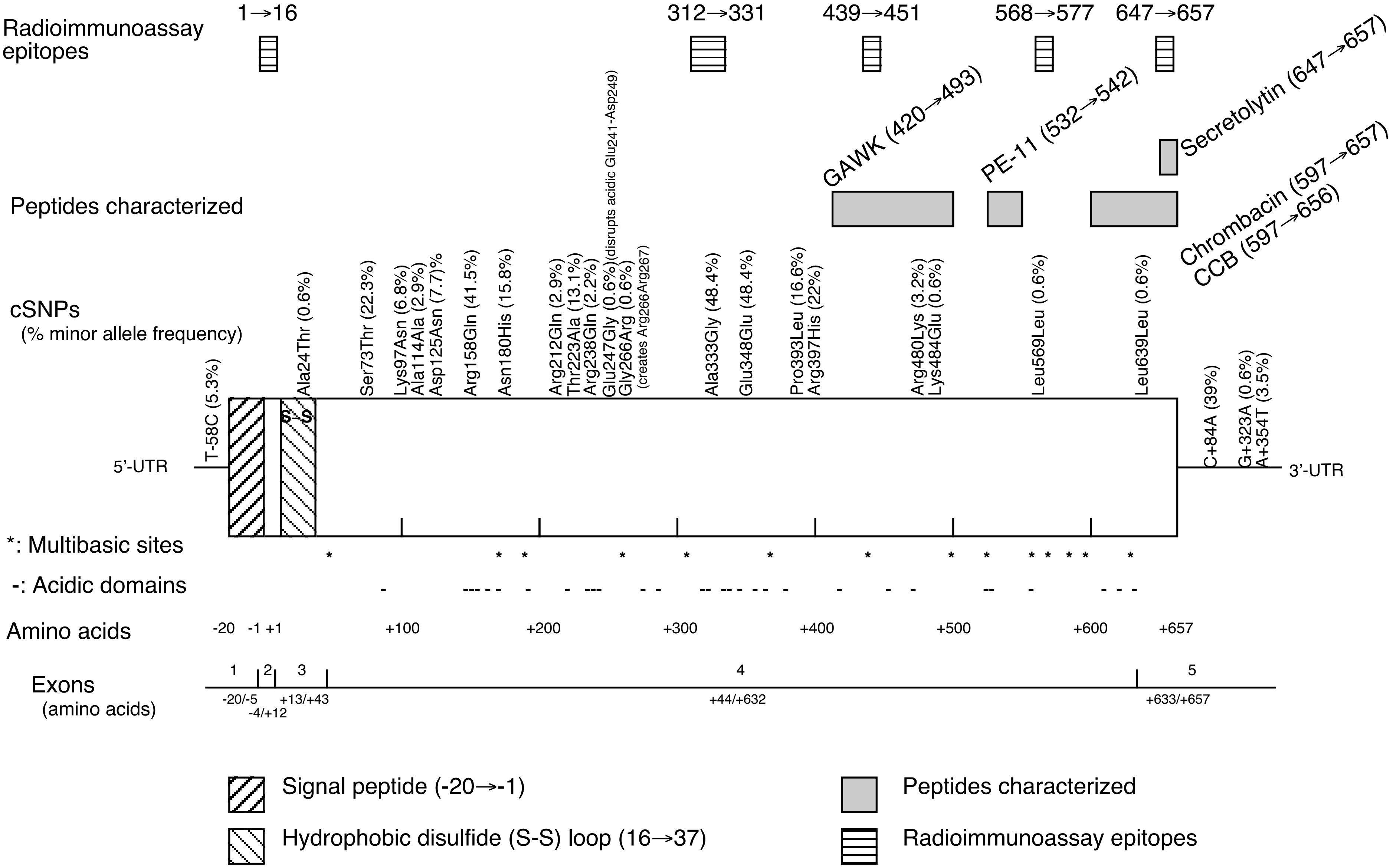
Human chromogranin B (CHGB):
Coding region cSNPs, functional domains, and epitopes



On-line supplementary Figure 3

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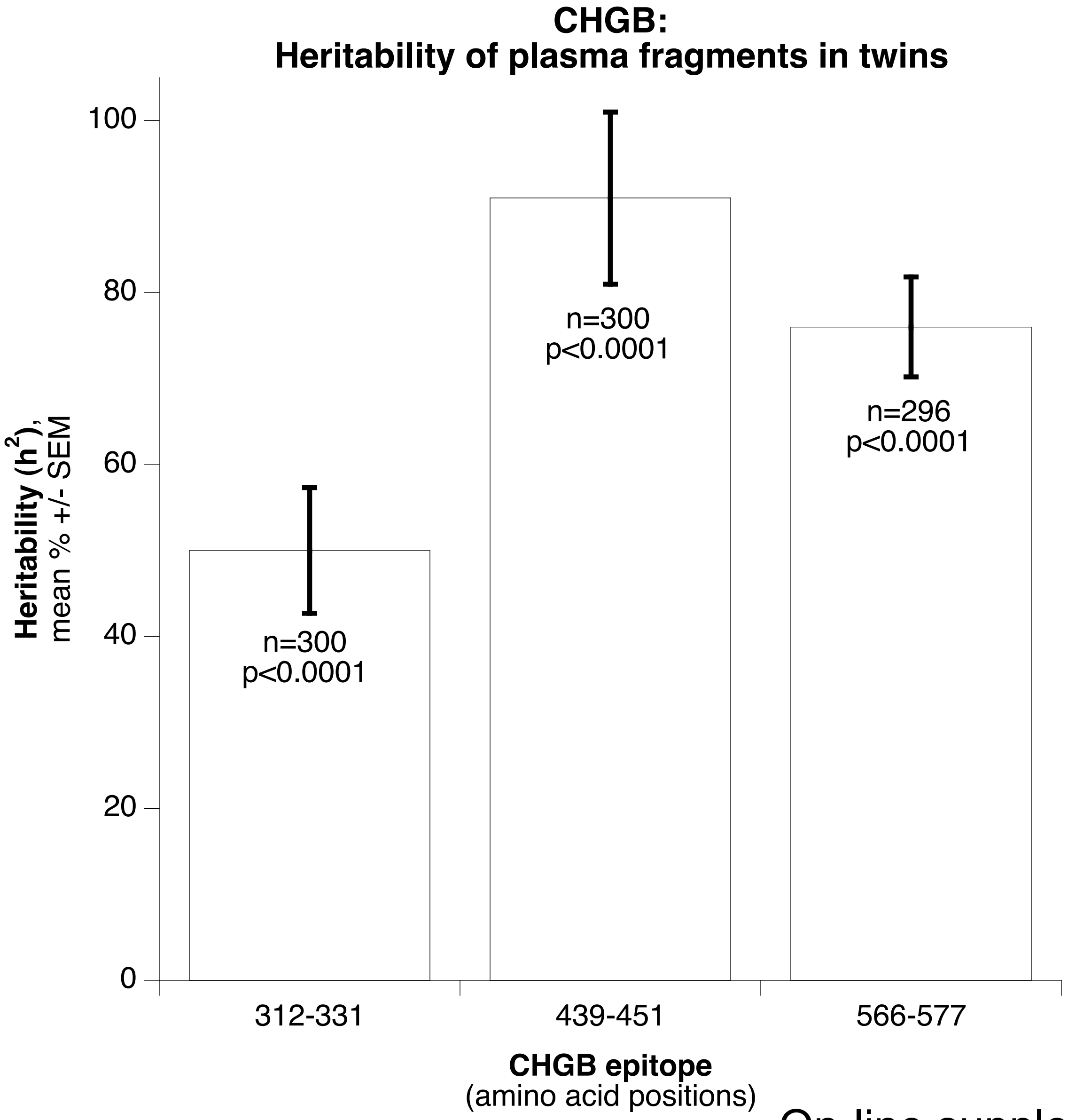

On-line supplementary Figure 4

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