Natural Variation within the Neuronal Nicotinic Acetylcholine Receptor Cluster on Human Chromosome 15q24: Influence on Heritable Autonomic Traits in Twin Pairs


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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) are combinations of subunits arranged as pentamers encircling a central cation channel. At least nine α and four β subunits are expressed in the central and peripheral nervous systems; their presence in autonomic ganglia, the adrenal medulla, and central nervous system, with accompanying responses elicited by nicotinic agonists, point to their involvement in cardiovascular homeostasis. nAChRs formed by α3, α5, and β4 subunits may regulate blood pressure (BP) by mediating release of catestatin, the endogenous nicotinic antagonist fragment of chromogranin A (CHGA) and potent inhibitor of catecholamine secretion. Genes encoding these subunits (CHRNA3, CHRNA5, and CHRN4) are clustered on human chromosome 15q24. Because variation in this cluster may alter autonomic regulation of BP, we sequenced ~15 kilobase pairs in 15q24 containing their coding and 5'- and 3'-untranslated regions in 80 individuals. We identified 63 variants: 25 in coding regions of CHRNA3, CHRNA5, and CHRN4 and 48 noncoding single-nucleotide polymorphisms (SNPs). Haplotype frequencies varied across ethnic populations. We assessed the contribution of six SNPs in the putative catestatin binding region of CHRNA3 and CHRN4 to autonomic traits. In twins, catestatin and BP were heritable. CHRNA3 SNPs and haplotypes containing K95K (G285A) associated with circulating plasma catestatin, epinephrine levels, as well as systolic BP, suggesting altered coupling of the nAChRs to BP. Studies of chromatin cells in vitro reveal that nicotinic agonist stimulation releases catecholamines and CHGA, a process augmented by overexpression of CHRNA3 and blocked by catestatin. These cellular events suggest a homeostatic mechanism underlying the pleiotropic actions of CHRNA3 genetic variation on autonomic function observed in twins.

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Abbreviations: nAChR, nicotinic acetylcholine receptor; CHGA, chromogranin A; CNS, central nervous system; ACh, acetylcholine; BP, blood pressure; SNP, single-nucleotide polymorphism; bp, base pair(s); EAP, embryonic alkaline phosphatase; h2, heritability; UTR, untranslated region; kb, kilobase(s); LD, linkage disequilibrium; SBP, systolic blood pressure; BW284c51, 1,5-bis (4-allyldimethylammoniumphenyl)-pentan-3-one dibromide.
the opening of voltage-gated Ca$^{2+}$ channels. Channel opening triggers exocytic transmitter or hormone release and synaptic transmission in autonomic ganglia and adrenal medullary chromaffin cells. The resulting catecholamine (epinephrine and norepinephrine) release is responsible for autonomic control of heart rate and peripheral vascular resistance (Goodman et al., 2005).

Whereas nAChR activation requires simultaneous occupation by more than one agonist molecule, a single competitive antagonist is sufficient to block function (Sine and Taylor, 1981). Various noncompetitive antagonists have been identified; they may exert inhibition by entering into the internal ion channel and restricting conformational changes associated with ion gating or restricting ion translocation (Karlin, 2002). Noncompetitive antagonists may perturb state transitions that accompany nAChR activation and desensitization by acting at other sites on the extracellular or transmembrane domains (Hansen and Taylor, 2007). In particular, the catabesin peptide fragment of the endogenous catecholamine secretory vesicle protein chromogranin A (CHGA) (human CHGA<sub>352–372</sub> and bovine CHGA<sub>344–364</sub>) acts as an antagonist. Its inhibition of nicotinic cationic channels (Mahata et al., 1997) may reduce catecholamine release; yet, its ability to prevent agonist-mediated desensitization might preserve such secretion in some circumstances (Mahata et al., 1999).

Neuronal nAChRs are found primarily in the CNS, peripheral autonomic ganglia, and adrenal medulla. These receptors are made up of pentameric combinations of α (α2–α10) and β (β2–β4) subunits, although only certain subunit combinations assemble into pentamers to confer function (Gotti et al., 2006). The α subunits are distinguished by vicinal cysteines on the C loop of the subunit. All α subunits, except α5, can form a principal face of the ligand-binding site. The complementary face is provided by the β subunits or, in some cases, α5.

Electron microscopy reconstruction analysis has yielded structures of the entire receptor enfolded in the membrane at ~4.5-Å resolution (Unwin, 2005). A particularly important model for ligand binding comes from the crystal structure of the ACh binding protein of fresh and salt water snails (Brejc et al., 2001). This protein serves as a structural surrogate for the extracellular domain of the nAChR because it has requisite specificity and rapid binding characteristics of nicotinic ligands, a sequence homologous to the extracellular domain of the receptor, and becomes functional when linked to the transmembrane regions of the receptor (Bouaz et al., 2004). High-resolution crystal structures provide a template for assigning the full complement of residues that form the ligand binding sites (Celie et al., 2004; Hansen et al., 2005) and enables localization of amino acid determinants in the three-dimensional structure of nAChR to infer the function of naturally occurring variants (genetic polymorphisms).

Genetic variants at a cluster of three genes (CHRNA3, CHRNB4, and CHRNA5) encoding nAChR subunits α3, β4, and α5 have gained attention recently. The cluster is located within a ~90-kilobase region of chromosome 15q24 in human and in a conserved area of synteny in other mammalian species (Boulter et al., 1990; Duga et al., 2001). The proximal locations of these genes suggest common regulation in the evolutionary process. Receptors containing α3 and β4 subunits, and possibly α5, are found in autonomic ganglia (Lindstrom, 2003). The presence of the gene product in peripheral and central sites makes them prime candidates for examining whether polymorphisms in nAChR subunit genes give rise to phenotypic manifestations expressed in the autonomic nervous system, in particular blood pressure (BP) regulation.

Recent genome-wide association studies report a role for nAChR subunit variants in heritable traits associated with smoking addiction and lung cancer risk (Berrettini et al., 2008; Hung et al., 2008; McKay et al., 2008; Thorgeirsson et al., 2008; Weiss et al., 2008). A common SNP of the α3 subunit, CHRNA3 rs1051730 (Y215F), is associated with nicotine dependence and confers risk for peripheral arterial disease and lung cancer in populations of European descent (Thorgeirsson et al., 2008). In our study, we undertook comprehensive sequencing of the entire nAChR cluster on human 15q24 to identify common variants and local patterns of linkage disequilibrium. Here, we examine the relationship between polymorphisms within this gene cluster and heritable autonomic traits in human twin pairs.

**Materials and Methods**

**Human Subjects.** Eighty unrelated subjects (34 Caucasian-Americans, 30 African-Americans, 8 Hispanics, 2 Chinese, and 6 Filipinos) were recruited from an urban area of San Diego, California. We recruited 438 twins of multiple ethnicities by accessing a population birth record-based twin registry (Cockburn et al., 2002) and a newspaper advertisement. Ethnicities of twin and unrelated subjects were established by self-identification, and ethnicities were reported for both parents and all four grandparents. The primary genetic analyses were conducted on 185 Caucasian-American twin pairs (370 individuals): 129 pairs were monozygotic (25 male, 104 female) and 56 pairs were dizygotic (10 male, 34 female, and 12 male/female). Twin zygosity assignments were based on self-identification and typing multiple SNP and microsatellite polymorphisms (Zhang et al., 2004). Twin ages were 15 to 84 years. Family histories for hypertension (in a first-degree relative before the age of 60 years) were as follows for the 185 pairs: 83 were positive (one or both parents), 87 were negative, and 15 were indeterminate/unknown. Three hundred thirty two individuals were normotensive and 38 were hypertensive (28 treated with antihypertensive medications; 10 untreated). None of the subjects had a history of renal failure. Subjects gave informed, written consent according to protocols approved by the University of California at San Diego Institutional Review Board. Genomic DNA was extracted from leukocytes using a PureGene DNA purification kit (Gentra Systems, Inc., Minneapolis, MN) according to manufacturer’s protocol. Primate DNA samples were obtained from Coriell Cell Repository (Camden, NJ).

**Sequencing and Genotyping Assays.** Oligonucleotide primers were designed to generate overlapping fragments [approximately 800 base pairs (bp)] of CHRNA3, CHRNA5, and CHRN4 (GenBank accession number NT_010194.16) (Supplemental Table S1 and Supplemental Fig. S1). Polymerase chain reaction and sequencing reactions were performed as described previously (Murthy et al., 2005). Twin samples were genotyped on a Pyrosequencing HSA96A instrument (Pyrosequencing AB, Uppsala, Sweden; www.pyrosequencing.com) as described previously (Murthy et al., 2005). The Phred, Phrap, and Consed suite of sequence analysis software was used to automate base calling, assemble sequence fragments, visualize sequence data, and detect heterozygous sites.

**Chromogranin Gene Expression in Vivo.** Plasma CHGA-derived peptide fragment levels were measured by radioimmunoassay of EDTA-anticoagulated plasma as described previously by Schildberg et al. (2004). Radiolabeling of each peptide was enabled by either an endogenous or adventitious (terminal) Tyr residue. Polyclonal rabbit antisera were developed to synthetic CHGA regions.
Human catestatin (CHGA352–372) was accessed by a radioimmunoassay using the CHGA361–372 epitope.

Physiological and Autonomic Phenotyping. Twin phenotyping was conducted prospectively, before genotyping. Blood pressure (mm Hg) and pulse interval (R-R interval or heart period; milliseconds/beat) were recorded continuously and noninvasively for 5 min in seated subjects with a radial artery applanation device and dedicated sensor hardware (Colin Pilot; Colin Instruments, San Antonio, TX) and software (ATLAS from WR Medical, Stillwater, MN; and Autonomic Nervous System/Tonometric Data Analysis (ANS/TDA), Colin Instruments), calibrated every 5 min against ipsilateral brachial arterial pressure with a cuff sphygmomanometer (Omron Colin Medical Corp., San Antonio, TX).

Acetylcholinesterase Activity in Blood. EDTA-anticoagulated, frozen whole blood samples were analyzed for cholinesterase activity on the erythrocyte surface using a modification of Ellman assay (Ellman and Callaway, 1961). Increase of absorbance at 436 nm was measured in diluted whole blood samples upon addition of substrate acetylthiocholine (0.5 mM). 5,5′-Dithio-bis(2-nitrobenzoic acid) (0.3 mM) was used as the chromogenic indicator of thiocholine formation. Samples were measured in 1-cm optical path cuvettes in a spectrophotometer (Gilford, Oberlin, OH) for 5 min at 37°C. Cholinesterase activity was recorded as mean changes in absorbance at 436 nm in 1 min per microliter of blood. Each sample was assayed for acetylcholinesterase activity by including the specific inhibitor BW284c51 at a final concentration of 1 μM. Activity was expressed as micromoles of acetylthiocholine hydrolyzed per microliter of sample per minute.

Plasma Catecholamine Measurements. Plasma catecholamines were measured radioenzymatically (Zhang et al., 2004). The assay uses a preconcentration step that increases sensitivity by ~10-fold over other catechol-O-methyltransferase-based assays and ~20-fold over many high-performance liquid chromatography assays, permitting accurate measurement of basal plasma epinephrine levels, which are at the limit of sensitivity for high-performance liquid chromatography assays.

Cellular Exocytosis Assays. Expression of the human CHGA/embryonic alkaline phosphatase (EAP) chimera was driven by an inserted cytomegalovirus promoter; this chimera is trafficked to chromaffin granules of the regulated secretory pathway, wherein the EAP reporter can be detected by a sensitive chemiluminescent assay (Taupenot et al., 2005). Expression of the mouse Chrna3 cDNA was driven by the cytomegalovirus promoter. PC12 chromaffin cells were cotransfected with these constructs, and chromaffin granule exocytosis was monitored by chemiluminescent assay of the EAP reporter.

Fig. 1. Top, homology model of the structure of the extracellular domain of the α3β4 nAChR derived from the structure of ACh binding proteins from Aplysia californica and Lymnaea stagnalis. Sequences for the extracellular domains for the α3 and β4 subunits are inserted and positioned by homology, after which the structure is energy minimized. The coding variants are shown in two views with nicotine. Left, synaptic apical position looking into the channel vestibule formed by the pentamer of subunits. Right, an α3β4 interface is viewed from the outer perimeter of the pentamic molecule. Amino acid residues representing the location of the non-synonymous coding variants are shown in yellow. Bottom, similar views of the ACh binding protein with the locations of α3β4 coding variants.
in the extracellular medium after secretory stimulation for 30 min by 60 μM nicotine in the presence or absence of 10 μM catemisin. Experiments were conducted in triplicate, and blank (mock) values were subtracted from stimulated secretion results.

**Statistical Analyses.** Descriptive statistics (mean ± S.E.M.) were computed across the twin sets, using generalized estimating equations (PROC GENMOD), in Statistical Analysis System (SAS Institute, Cary, NC), to take into account intratwin-pair correlations. Estimates of heritability (h² = Vp/Vc, where Vc is additive genetic variance and Vp is total phenotypic variance) were obtained using the variance-component methodology implemented in the SOLAR package (Almasy and Blangero, 1998). This method maximizes the likelihood assuming a multivariate normal distribution of phenotypes in twin pairs (monozygotic versus dizygotic), with a mean dependent on a particular set of explanatory covariates. The null hypothesis (H0) of no heritability was tested by comparing the full model, which assumes genetic variation (Vp), and a reduced model, which assumes no genetic variation, using a likelihood ratio test. All heritability estimates were adjusted for age and sex, because of the effects of these covariates on several traits (Zhang et al., 2004). GOLD and Haploview were used to estimate D' between SNPs (Abecasis and Cookson, 2000; Barrett et al., 2005).

**Results**

**Polymorphism Discovery.** We sequenced the coding regions, untranslated regions (UTR), approximately 600 bp of promoter, and approximately 100 bp of exon-flanking intronic regions of the CHRNA3, CHRNA5, CHRNA4 genes contained within the 76-kb region of the nAChR cluster in 80 unrelated and ethnically diverse individuals. We identified 20 SNPs, five insertion/deletion polymorphisms, and one microsatellite repeat from sequencing 5404 bp of CHRNA3, 20 of the biallelic variants were identified in more than one individual with >1% frequency. In the 4988 bp of CHRNA5 sequenced, 25 biallelic genetic variants (SNPs and insertion/deletion) were identified, of which 19 were identified in more than one individual. In the 4513-bp sequenced region of CHRNA4, we identified 29 biallelic markers, of which 15 were observed in more than one individual. The variants and their frequencies in each population are listed in Supplementary Table S2.

**Coding Region SNPs.** Within the coding regions of the nAChR cluster, we identified 25 SNPs: 10 nonsynonymous (coding region SNPs) and 15 synonymous (Supplementary Table S3). As expected, there were a greater number of synonymous sites, and these also had higher minor allele frequencies than the nonsynonymous sites. The amino acid

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Fig. 2. Major haplotypes across the nAChR cluster (CHRNA3, CHRNA5, and CHRNA4) in Caucasian- and African-Americans. Haplotype frequencies were determined from sequence information on 64 Caucasian-American (C-A) and 60 African-American (A-A) chromosomes, using 26 common variants. SNPs in order (5’ to 3’) are CHRNA5_1 = 5’-UTR [-219 to -240]; CHRNA5_2 = 5’-UTR [-160]T/A; CHRNA5_3 = 5’-UTR [-123]T/G; CHRNA5_4 = 5’-UTR [-76]C/G; CHRNA5_5 = IN1[-34]CT; CHRNA5_6 = IN3[+211]G/A; CHRNA5_7 = 3’-UTR [+395]CT; CHRNA5_8 = 3’-UTR [+603]C/T; CHRNA5_9 = 3’-UTR [+632]A/G; CHRNA3_1 = 3’-UTR [+1237] to 1240]G(A); CHRNA3_2 = 3’-UTR + 1114C→T; CHRNA3_3 = 3’-UTR + 952C→T; CHRNA3_4 = 3’-UTR + 708 In(GGGG); CHRNA3_5 = 3’-UTR + 163 Del(A); CHRNA3_6 = 639C→T; CHRNA3_7 = 285G→A (R95K); CHRNA3_8 = IN3[+5]C→T; CHRNA3_9 = 153A→G (V51V); CHRNA3_10 = 105G→A (B35H); CHRNA4_1 = 3’UTR [+76IC/T; CHRNA4_2 = IN5[+220]C/G; CHRNA4_3 = 420A/G; CHRNA4_4 = 272CT/T (T91I); CHRNA4_5 = IN2[+192]A/C; CHRNA4_6 = 122A/G; CHRNA4_7 = 5’-UTR [-843]G/C.
substitutions were localized to positions in the overall structure of ACh binding protein for the extracellular domain (Hansen et al., 2005; Hansen and Taylor, 2007) and for the lower resolution structure of the nAChR for the transmembrane and cytoplasmic regions (Unwin, 2005) (Fig. 1).

Population Differences in Allele Frequencies. The number and distribution of polymorphic sites differed among ethnic groups, and several variants were observed to be population specific (Supplemental Table S2). The African-American population had a greater number of variable sites than Caucasian-Americans for all three genes, although the ratio of the number of variable sites in the African-Americans to Caucasian-Americans differed among the three genes. Common SNPs such as CHRNA3 V51V (31% minor allele frequency) and CHRNA3 IN3[−5]A/C (28% minor allele frequency) were identified in all populations examined. In contrast, the nonsynonymous SNP CHRNA5 L363Q was common in the African-Americans (9%) and observed only in this population. CHRN412951 was polymorphic in African-Americans but was not observed in Caucasian-Americans; this SNP was also observed in Hispanics and Asians despite the relatively fewer numbers of individuals examined in these two groups.

Haplotype Analysis and Linkage Disequilibrium Pattern. Twenty-six variants at the three loci with allele frequencies >10% in the unrelated individuals were used to assess pairwise LD patterns in the 76-kb region containing the nAChR gene cluster and estimate frequencies of common haplotypes in the Caucasian- and African-American populations (Fig. 2). SNPs within CHRNA3 and CHRNA4 were in strong LD with each other in the Caucasian population, with two common SNPs at CHRNA3 (K95K and IN3[−5]C→T) in almost complete LD (D' = 0.94). Common haplotypes of the Caucasian-American population were in lower frequency in the African-Americans, and several haplotypes identified in African-Americans were not observed in Caucasian-American population specific (Supplemental Table S2). The African-American

**Fig. 3.** Haplotype phylogeny across CHRNA3 and CHRNA4 on chromosome 15q24. Genotyping and haplotype inference on 370 twin samples suggest that “common” and “rare” haplotypes were derived from two ancestral haplotypes, A and V, through either recombination events (dashed arrows) or mutation events (solid arrows). The inferred “ancestral” haplotype A is still observed in the population today; however, another potential ancestral haplotype (V) may have existed, but the identity of SNP position 3 (indicated by ■) cannot be predicted with available data. The relative position of SNPs in exons (numbered boxes) and introns of CHRNA3 and CHRNA4 are diagrammed with the size of introns labeled below intron the number; gene structure is not drawn to precise scale.

<table>
<thead>
<tr>
<th>Phenytype</th>
<th>Mean ± S.E.M.</th>
<th>h^2 (± S.E.M.*)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>40.6 ± 0.9</td>
<td>83.0 ± 2.5</td>
<td>93.4</td>
</tr>
<tr>
<td>Body mass index (kg/m^2)</td>
<td>25.0 ± 0.3</td>
<td>34.8 ± 8.5</td>
<td>68.2</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>116.8 ± 0.9</td>
<td>30.4 ± 7.6</td>
<td>68.2</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>63.4 ± 0.6</td>
<td>14.8 ± 8.5</td>
<td>68.2</td>
</tr>
<tr>
<td>Plasma catestatin</td>
<td>1.30 ± 0.04</td>
<td>64.7 ± 6.2</td>
<td>68.2</td>
</tr>
<tr>
<td>Plasma chromogranin B_361-372 [nM]</td>
<td>0.39 ± 0.01</td>
<td>67.1 ± 5.3</td>
<td>68.2</td>
</tr>
<tr>
<td>Erythrocyte acetylcholinesterase activity [dA/min/μl]</td>
<td>42.9 ± 1.3</td>
<td>61.4 ± 9.8</td>
<td>68.2</td>
</tr>
<tr>
<td>Plasma epinephrine [ng/ml]</td>
<td>315 ± 11.6</td>
<td>55.5 ± 8.0</td>
<td>68.2</td>
</tr>
</tbody>
</table>

* Adjusted for age and sex; scaled from 0 to 100.
cans. Haplotype associations with SBP are shown in Table 2. The four CHRNA3 SNPs genotyped in the twin study were sex-limited in their association with SBP: CHRNA3 (IN3–5C; IN2) and CHRNA3 (IN2–5C; T) showed significant (p = 0.016) and nearly significant (p = 0.019) associations with SBP, respectively. CHRNA3 (IN3–5C; T) showed a trend for increased SBP, whereas haplotype C was associated with decreased SBP (p = 0.0195) with increased plasma circulating CHG361–372 levels of 0.9, 1.28 ± 0.06, and 1.05 ± 0.11 nM, respectively. The joint (or pleiotropic) effect of CHRNA3 K95K on both catestatin and SBP suggests a change in the coupling of the peptide’s actions toward blood pressure (Fig. 4b). The T allele of CHRNA3 (IN3–5C; T) was also significantly associated (p = 0.012) with increased plasma circulating CHG361–372 levels: twins with T/T (n = 144), T/C (n = 170), and C/C (n = 52) genotypes had average circulating CHG361–372 levels of 1.42 ± 0.09, 1.28 ± 0.06, and 1.05 ± 0.11 nM, respectively. The T allele of CHRNA3 (IN3–5C; T) showed a trend for elevated SBP but did not reach significance (p = 0.059). We found no nAChR SNP associations with body mass index, blood pressure, or recombination events (haplotypes F, I, L, and K), whereas ancestral haplotype V gave rise to the more common haplotypes observed in our twin population. The one haplotype observed in the three chimpanzees was not present in our twin population.

**Twin Pairs: Haplotype Phylogeny.** We genotyped 370 twin subjects at six common SNPs identified in this study that might play a role in influencing the vestibule shape or proteinase of the a3-4 nictinic receptor, or otherwise “tag” LD blocks: CHRNA3 (285G; K95K), CHRNA3 (IN3–5C; T), CHRNA3 (R35H), CHRNA3 (153A; G, V51V), CHRN4B (IN2–192A; C), and CHRN4B (T91I). Haplotype inferences on these data were conducted to enable haplotype-based association analyses with cardiovascular phenotypes. The block partition method for haplotype inference (Halperin and Eskin, 2004) (Fig. 3) identified two ancestral haplotypes (A and V) from which subsequent haplotypes were derived through mutation or recombination events: haplotype A contains an adenine (A) nucleotide at CHRNA3 (K95K) and a cytosine (C) nucleotide at CHRNA3 (IN3–5C; T); haplotype V contains the opposite allele at these two SNPs. Ancestral haplotype A potentially gave rise to the rare haplotypes observed in our population through mutation events (haplotypes H and J) or recombination events (haplotypes F, I, L, and K), whereas ancestral haplotype V gave rise to the more common haplotypes in our population (B and C) observed in both Caucasian- and African-Americans. Comparison of haplotypes in chimpanzee and gorilla at these six polymorphic sites (Fig. 2) revealed that the observed gorilla haplotype is identical to the common human haplotype B. The one haplotype observed in the three chimpanzees was not present in our twin population.

**Twin Pairs: Trait Association Studies.** We conducted genetic association analyses on the 370 Caucasian-American twin pairs for BP and heritable intermediate traits for hypertension probably influenced by autonomic nAChRs; trait heritabilities are listed in Table 1. Although BP displays significant heritability (h2 of ~30–35%), the plasma concentrations of catestatin (epitope: CHG361–372) and CHGB (epitope: CgB39–451) are substantially more heritable (h2 of ~50–65%). The four CHRNA3 SNPs genotyped in the twin study were sex-limited in their association with SBP: CHRNA3 (IN3–5C; T) and CHRNA3 (IN2–5C; T) were significantly associated with SBP (p = 0.016) in a pattern indicative of an additive effect of this allele in lowering SBP (Fig. 4a): the average SBP of twins with genotype G/G is 119.5 ± 1.5 mm Hg (n = 140), G/A is 116.3 ± 1.5 mm Hg (n = 168), and A/A is 112.9 ± 1.7 mm Hg (n = 52). The A allele of CHRNA3 (K95K) was also significantly (p = 0.012) associated with decreased plasma circulating catestatin (CHG361–372) levels: twins with G/G, G/A, and A/A genotypes had declining average circulating CHG361–372 levels of 1.40 ± 0.09, 1.27 ± 0.056, and 1.09 ± 0.09 nM, respectively.

The joint (or pleiotropic) effect of CHRNA3 K95K on both catestatin and SBP suggests a change in the coupling of the peptide’s actions toward blood pressure (Fig. 4b). The T allele of CHRNA3 (IN3–5C; T) was also significantly associated (p = 0.012) with increased plasma circulating CHG361–372 levels: twins with T/T (n = 144), T/C (n = 170), and C/C (n = 52) genotypes had average circulating CHG361–372 levels of 1.42 ± 0.09, 1.28 ± 0.06, and 1.05 ± 0.11 nM, respectively. The T allele of CHRNA3 (IN3–5C; T) showed a trend for increased SBP but did not reach significance (p = 0.059). We found no nAChR SNP associations with body mass index, blood pressure, or recombination events.
site as well as the CHRNA3 (IN3[−5]C→T) site, did not reveal association with either SBP or catestatin.

Nicotinic AChRs and the Catestatin Phenotype: Cellular Mechanism. To probe the relationship between nAChR stimulation and catestatin, we studied exocytotic secretion, expressed from an in-frame CHGA/EAP chimera, trafficked to chromaffin granules within chromaffin cells (Taupenot et al., 2005), and the effects of added/exogenous Chrna3 (α3-containing receptors), with stimulation by a nicotinic agonist in the presence or absence of catestatin blockade of nicotinic cholinergic transmission (Fig. 5). The gene product of the CHGA/EAP chimera was secreted by PC12 cells, and the secretion was augmented ~4-fold by increasing the expression of α3 subunits, thus establishing the potential for quantitative changes in CHRNA3 expression to alter exocytosis traits. Exocytosis was increased from ~1.4- to 2.9-fold by nicotinic agonist stimulation, and the nicotine-elicited increments were completely blocked by the CHGA fragment catestatin. Even in the absence of nicotine, the CHGA/EAP displayed some basal secretion by PC12 cells, perhaps reflecting endogenous (autocrine) acetylcholine production/secretion, as described previously for this cell line (Roghani and Carroll, 2002). Consistent with this viewpoint, even the nicotine-independent secretion was completely blocked by catestatin.

Discussion

Putative Functional SNPs of the nAChR α3, α5, and β4 Subunit Genes Identified through Population Sequencing. We mapped coding region SNPs to their amino acid residue positions on nAChR using an ACh binding protein-based in silico-generated representation of nAChR to infer their functional impact. For example, β4 (I66F), identified in one individual, localized in the central vestibule of the heterodimeric nAChR. In silico modeling suggests that a change from the aliphatic Ile to the aromatic Phe at this residue could alter open channel conductance because rings of anionic charges are located within the vestibule as well as at the extracellular entry point of the transmembrane span (Hansen et al., 2008). The more common variants with putative functional impact such as α3(R35H) were genotyped in twins for association with hypertension.

Noncoding SNPs were identified in gene regions that may influence gene expression. For example, CHRN4 (IN3[+1]G→A) identified in one individual alters the GT/AG splice donor site and likely splicing efficiency. This rare SNP was not included in the twin study, but the more common CHRNA3 (IN3[−5]C→T) SNP, located only five nucleotides upstream from the intron 3-exon 4 junction, was genotyped. The significance of this pyrimidine-to-pyrimidine change (C→T) in the conserved polypyrimidine tract-acceptor splice site of the intron is difficult to determine through informatics approaches alone (Sverdlov et al., 2004). The consensus splice acceptor site may or may not be altered by this variant, and further molecular approaches and association studies on this SNP are needed to reveal its significance.

Association with Intermediate Traits for Hypertension. The primary phenotype of hypertension, elevated BP, is obscured by a variety of environmental influences, physiological factors controlling cardiac output and peripheral vascular resistance. The late age of onset (late penetrance) of hypertension is an added complexity. These issues pose an obstacle to identify genetic contributors. Association studies using simpler, more heritable intermediate traits to hypertension that can be elicited in still-normotensive individuals can resolve these issues (O’Connor et al., 2000; Kato, 2002). Intermediate traits using a twin sampling design is a powerful strategy for genetic association studies and can point to testable candidate genes (Martin et al., 1997; Luft, 2001).

Using this approach, we detected contribution of several common CHRN4 and CHRN8 variants and their haplotypes to hypertension intermediate traits involved in the nAChR pathway.

We found a significant contribution of the CHRNA3 K95K (A) allele (rs3743075) and the CHRNA3 (IN3[−5]C→T) C allele to circulating levels of catestatin (CHGA361→T72). We propose that individuals with this combination of SNPs may have higher receptor affinity for the endogenously generated peptide, thus decreasing the detectable levels of secreted catestatin in the plasma, perhaps by altered negative feedback inhibition. Indeed, a two-dimensional plot of the effects of K95K on catestatin and SBP (Fig. 4b) suggests an alteration in the coupling between catestatin and BP, a relationship made apparent after stratification by K95K genotype. In this setting, minor allele homozygosity (A/A) results in lower SBP even at lower circulating catestatin concentration. Furthermore, directionally coordinate changes in SBP and epinephrine as a function of the K95K genotype (Fig. 4e) were observed and suggest a mechanism for the effects of CHRNA3 genetic variation on BP: alteration in nicotinic control of catecholamine release initiates changes in basal BP. Intriguingly, G/G homozygotes, who express higher plasma catestatin levels, displayed elevated plasma epinephrine and BP (Fig. 4e). This finding was unexpected because catestatin has been described as a nicotinic cholinergic antagonist to diminish catecholamine release (Mahata et al., 1997). However, three lines of evidence may be pertinent to
this observation. First, prolonged exposure to catestatin may block nicotinic cholinergic tolerance/desensitization, paradoxically inhibiting catecholamine release (Mahata et al., 1999). Second, studies on catestatin variants indicate that the peptide may act on CNS (probably in the nucleus of the tractus solitarius) nicotinic synapses to inhibit catecholamine release, consequently increasing BP (Rao et al., 2007). Third, stratification by CHRNA3 genotype may reveal directionally coordinate effects of this trans-quantitative trait locus on multiple autonomic traits.

Although catestatin at nanomolar circulating concentrations paralleled SBP when stratified by CHRNA3 genotype, the typical catestatin concentration to inhibit nicotinic cholinergic stimulation of cultured chromaffin cells is EC$_{50}$ ~ 200 nM (Mahata et al., 1997); thus, rather than exerting systemic/endocrine actions, the endogenous responses to catestatin may reflect autocrine/paracrine release in the immediate vicinity of chromaffin cells, thereby acting locally to control exocytotic events. In addition, CHRNA3 genotype stratification uncovered a direct parallel between circulating catestatin and SBP. Because the CNS and peripheral nervous system responses to nicotinic stimulation and catestatin may be different (indeed, opposing each other), the CHRNA3/catestatin/SBP direct coupling (Fig. 4b) may suggest that the catestatin/nicotinic actions underlying BP effects in this setting may be more central than peripheral (Rao et al., 2007).

A recent genome-wide association scan found that the CHRNA3 (Y215Y) SNP (rs1051730) correlates with nicotine dependence and smoking quantity (Thorgeirsson et al., 2008). In other studies, this SNP also conferred risk for lung disease and cardiovascular disease through enhancing nicotine dependence (Hung et al., 2008; McKay et al., 2008; Thorgeirsson et al., 2008). We assessed whether our observations are due to smoking behavior, rather than directly through the effenter autonomic pathway. Cigarette smoking did indeed affect SBP in our 438 twins ($p$ = 0.03); however, the effect of the K95K SNP on SBP survived adjustment for smoking, remaining at $p$ = 0.01. Thus, smoking behavior did not confound association of the K95K SNP with circulating plasma catestatin or epinephrine levels.

We demonstrated an association to both SBP and circulating epinephrine with the K95K SNP but not the Y215Y SNP. The K95K and Y215Y SNPs are just over 5 kb apart in CHRNA3, and significant LD of SNPs within the nAChR gene cluster region has been reported (Hung et al., 2008). We found these two SNPs to be in high LD ($D' = 0.89$) in our Caucasians. Although all three possible combinations of the SNPs were observed, haplotype K95K(G)$\rightarrow$Y215Y(T) was most common. Noteworthy is that the K95K(G) allele, associated with increased SBP and plasma epinephrine, occurs on the common haplotype containing the peripheral arterial disease risk conferring allele, Y215Y(T), of previous reports. The Y215Y(T) allele is at lower frequency (0.19) than the K95K(A) allele (0.48) in Caucasians, thus the K95K SNP may have greater statistical power to capture associations by virtue of LD. A larger sample size may allow detection of a contribution of the Y215Y SNP to BP traits. Alternatively, both SNPs may be in LD with yet another causative SNP such as those identified in the promoter, introns, or 3'-UTR. It is unclear whether the reported genome-wide association scan included K95K; the absence of K95K may explain why previous studies found significant association with Y15Y rather than K95K, despite K95K exhibiting higher minor allele frequency.

Haplotype association tests revealed that individuals possessing one or two copies of the very common ancestral haplotype A had both decreased SBP and circulating CHGA_361–372 levels, once again suggesting altered coupling between catestatin and BP. Haplotype A includes K95K(A) and CHRNA3 (IN3[5C$\rightarrow$T](C), which was associated with lower SBP and CHGA_361–372. Haplotype C, carrying the opposite alleles at these two sites, increased SBP (Fig. 4c). Parenthetically, haplotype B, which contains the same alleles at the CHRNA3 (K95K) and (IN3[5C$\rightarrow$T]) sites as haplotype C, but varies at CHRNA3 V51V from both haplotypes C and A, did not contribute to phenotypic variation of either catestatin or BP. Closer examination of our haplotype inference data using the global population and 26 SNPs ranging across the entire nicotinic receptor cluster reveals that haplotype B probably carries several other alleles not found on haplotypes A and C that may influence autonomic phenotypes.

**Cellular Mechanisms Controlling BP.** Studies in transfected chromaffin cells indicated that quantitative variation in murine Chrna3 expression can influence secretion of CHGA, the process is augmented by nicotine, and the CHGA fragment catestatin can inhibit these actions. Although associated with plasma catestatin and BP, K95K is synonymous and may not confer a qualitative or structural change in the α3 subunit; K95K probably acts as a surrogate for other nearby polymorphism(s) in LD. Because we also identified nearby SNPs in an intron-exon splice junction and the UTRs of CHRNA3, the mechanism of effect of K95K may be a quantitative change in the expression of CHRNA3, a circumstance that we have simulated in the chromaffin cell transfection experiments of Fig. 5. Murine Chrna3 overexpression increased CHGA/EAP chimera secretion, and the nicotinic-agonist evoked increase in chimera release was inhibited by catestatin. Although we have not yet determined the precise causal variant in the Chrna3 region, these cellular results suggest a hypothesis unifying our experimental observations as reported previously (O’Connor et al., 2008): Nicotinic agonist stimulation of nAChRs on chromaffin cells releases catestatin, which in negative feedback manner blocks nicotinic secretion. Augmented expression of the crucial α3 subunit of the nAChR affecting autonomic function (CHRNA3) may increase the gain of the system, thereby changing the coupling between catestatin modulation of nAChRs and BP.

We conclude that common genetic variants of the nAChR cluster on 15q24 contribute to interindividual variation in autonomic function and thus may represent risk factors for development of cardiovascular disease. Investigation of cellular nicotinic cholinergic mechanisms in chromaffin cells in vitro suggests a plausible feedback mechanism for the associations we observed in vivo. Replication of our findings in other large cohorts is required to determine the role of nAChR variants in hypertension.

**References**


