

# CHRNA4 and Tobacco Dependence

## From Gene Regulation to Treatment Outcome

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**Context:** Given the probable importance of the  $\alpha 4$  subunit of the neuronal nicotinic acetylcholine receptor, the gene that codes for this subunit (*CHRNA4*) represents an excellent starting point for a genetic investigation of smoking behavior.

**Objective:** To achieve a better understanding of the role of this gene in the cause and treatment of tobacco dependence, we adopted a transdisciplinary pharmacogenetic approach.

**Design:** Study at the behavioral and clinical levels of analysis.

**Setting:** Academic research.

**Participants:** Smokers (n=316) between the ages of 18 and 50 years were recruited from the Denver, Colorado, metropolitan area.

**Main Outcome Measures:** Bioinformatics analyses, cell culture experiments, and analyses of *CHRNA4* ex-

pression and nicotine binding in postmortem human brain tissue advanced 2 single-nucleotide polymorphisms (rs6122429 and rs2236196).

**Results:** Both single-nucleotide polymorphisms were associated with subjective responses to smoking in the laboratory among 316 smokers. Likewise, among 353 participants in a clinical trial, rs2236196 was associated with smoking cessation outcomes.

**Conclusions:** Results of analyses ranging from basic biological approaches to clinical outcome data provide consistent evidence that 2 single-nucleotide polymorphisms in *CHRNA4* are functional at a biological level and are associated with nicotine dependence phenotypes. This interdisciplinary approach to the genetics of nicotine dependence provides a model for testing how functional genetic variation is translated from changes in messenger RNA and protein to individual differences in behavior and treatment outcome.

*Arch Gen Psychiatry.* 2007;64(9):1078-1086

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**A**LTHOUGH RATES OF TOBACCO use have decreased nationally, it has been suggested that tobacco use is becoming a more intractable problem among the subset of the population who continue to smoke, making further reductions in use more difficult.<sup>1-4</sup> Greater understanding of individual differences in the cause and maintenance of tobacco use is necessary to develop more effective tobacco use reduction strategies. Research on the heritability of tobacco dependence leaves no doubt regarding a substantial role for genetic factors in the cause and maintenance of tobacco use,<sup>5</sup> and recent studies<sup>6,7</sup> identified specific genes that influence tobacco dependence.

One of the primary mechanisms underlying the addictive nature of tobacco use is the binding of nicotine to nicotinic

acetylcholine receptors. The  $\alpha 4$  subunit of the nicotinic receptor is highly expressed in the central nervous system (CNS) and plays a major role in tolerance, reward, and the modulation of mesolimbic dopamine function, all of which are critical to the development of tobacco dependence.<sup>7</sup> Given the central role of the  $\alpha 4$  subunit, the gene that expresses this subunit (*CHRNA4*) is a prime target for research into the genetic factors that influence tobacco dependence. To our knowledge, only 2 studies<sup>8,9</sup> have examined *CHRNA4* in humans, and both reports suggested a link between *CHRNA4* and tobacco use. However, neither study offered any evidence regarding the biological plausibility of the associations. Studies<sup>7,10,11</sup> in animals documented the importance of *CHRNA4* and mutations in *CHRNA4*. Finally, as further evidence of the relevance of *CHRNA4*, investigators noted associations between

*CHRNA4* and autosomal dominant nocturnal frontal lobe epilepsy,<sup>12-14</sup> attention-deficit/hyperactivity disorder,<sup>15,16</sup> Alzheimer disease,<sup>17</sup> and febrile convulsions.<sup>18</sup>

Reports of a genetic association often lack mechanistic translational data (ie, how a genetic variation may lead to changes at the RNA or protein level, which in turn lead to downstream changes in intermediate biological and behavioral phenotypes, which in turn lead to a change in the cause or treatment of tobacco dependence). A lack of translational data makes it difficult to understand how genetic variation may affect the neurobiology of tobacco dependence. The present investigation adopted a translational approach that spans multiple levels of analysis, including bioinformatics, cell culture models, human laboratory models, analyses of treatment outcome data, and protein expression in postmortem brain tissue. The first step in this “bottom up” approach was to advance single-nucleotide polymorphisms (SNPs) in *CHRNA4* using bioinformatics and cell culture methods and to test these SNPs for biological function using human postmortem brain tissue assays. The second step was to examine the effects of biologically functional SNPs on intermediate human phenotypes in the laboratory (eg, sensitivity to the effects of nicotine). Intermediate phenotypes (also known as *endophenotypes*) provide a vital mechanistic link in the translation of genomic variation to variation in the cause and treatment of behavioral disorders and have proved to be useful in previous association studies.<sup>19,20</sup> Finally, the third step was to examine the effects of these SNPs on dependence level and response to nicotine therapy in a large clinical trial.

## METHODS

### BIOINFORMATICS

The National Center for Biotechnology Information database of SNPs was searched for SNPs that were validated and could potentially alter transcription factor binding sites within the promoter of *CHRNA4*. Single-nucleotide polymorphisms in the 3' untranslated region (UTR) were screened for protein binding sites using the *CHRNA4* messenger RNA (mRNA) sequence provided in the National Center for Biotechnology Information accession AB209359. AB209359 was used because it includes the entire 3' UTR. Single-nucleotide polymorphisms that were identified as potentially functional were then advanced for testing using mobility shift assays and luciferase activity assays.

### MOBILITY SHIFT ASSAYS

Single-strand oligonucleotides flanking each SNP and containing both alleles of each SNP were obtained (Integrated DNA Technologies, Inc, Coraville, Iowa). The complementary strands were annealed and end labeled. Binding reactions were completed and then run on a 4% acrylamide gel in Tris, boric acid, and EDTA buffer for 60 minutes at 400 V.

### IN VITRO LUCIFERASE ACTIVITY ASSAYS

“Sensor” constructs containing 4 tandem copies of each version of the promoter SNP advanced from the mobility shift assay were placed upstream of a minimal 100–base pair (bp) thy-

midine kinase promoter driving the luciferase gene. These oligonucleotide sensor constructs were designed to contain 4 copies of the SNP with 12 bp flanking either side of the SNP, along with restriction sites on either end. Neuronal SH-SY5Y cells were used, and the experiments were performed using 3 to 8 wells per construct and were run 3 to 5 times for statistical purposes.

For the 3' UTR SNP, the 3' UTR was cloned using each version of the SNP and was placed downstream of the luciferase gene, which was driven by a minimal 100-bp thymidine kinase promoter. Neuronal SH-SY5Y cells were used for transfection. Experiments were performed using 3 to 8 wells per construct and were run 3 to 5 times for statistical purposes.

### GENOMIC DNA EXTRACTION AND SNP GENOTYPING

For the behavioral analyses, genomic DNA was collected from buccal cells (cheek swabs) following published procedures.<sup>21-23</sup> For the brain samples, genomic DNA was extracted from 5 to 10 mg of the prefrontal cortex using a commercially available kit (DNeasy Mini Kit; Qiagen, Valencia, California) according to the manufacturer's recommendations. For SNP rs6122429, the genomic DNA sequence (including the SNP) was submitted to a commercially available system for design and synthesis of a custom assay (ABI 7500; PE Biosystems, Foster City, California); the sequences for this assay are forward primer 5'-GGTCTCTGCGGAGGTACCA-3', reverse 5'-AGCCGTTTCAGGACTCTCTGT-3', the VIC probe 5'-TGCAGCCCCCGCCT-3', and the FAM probe 5'-TGCAGCCCCTGCCT-3'. Single-nucleotide polymorphism assays were performed per the manufacturer's recommendations using the real-time polymerase chain reaction instrument (ABI 7500).

### $\alpha 4\beta 2$ BINDING ASSAY IN HUMAN POSTMORTEM BRAIN TISSUE

For 20 subjects, 50 to 100 mg of nucleus accumbens tissue was obtained from the Australian Brain Donor Programs, New South Wales Tissue Resource Centre. Of these 20 subjects, all were men of white race/ethnicity.

To prepare crude particulate fractions, samples were thawed, suspended in 0.1× buffer (1× buffer comprises 140mM sodium chloride, 1.5mM potassium chloride, 2.0mM calcium chloride, 1.0mM magnesium sulfate, and 25mM HEPES [pH 7.5]), homogenized using a motor-driven pestle, and centrifuged at 20 000g for 20 minutes. Supernatants were discarded, and the pellets were resuspended in fresh hypotonic buffer and again centrifuged.

The binding of epibatidine labeled with iodine I 125 was measured as described previously.<sup>24</sup> Because <sup>125</sup>I-epibatidine binds with high affinity to several different nicotinic acetylcholine receptors, differential inhibition by the agonist cytosine (50nM and 150nM) was used to distinguish between cytosine-sensitive ( $\alpha 4\beta 2$  nicotinic acetylcholine receptors) and cytosine-resistant (non- $\alpha 4\beta 2$  nicotinic acetylcholine receptors) populations. Protein levels were determined using the method of Lowry et al.<sup>25</sup>

### BEHAVIORAL OUTCOMES

Smokers (n=316) between the ages of 18 and 50 years were recruited from the Denver, Colorado, metropolitan area. At a baseline session, participants provided informed consent and completed a battery of self-report tobacco use assessments. A demographic questionnaire was used at baseline to collect information on age, sex, income, education, occupation, marital

status, race/ethnicity, and socioeconomic status. A smoking history questionnaire was used to collect information on frequency and quantity of tobacco use. The Fagerström Test for Nicotine Dependence was used as a measure of nicotine dependence. Participants were then scheduled for the first experimental session 1 week later. Expired carbon monoxide levels were measured to verify 8-hour smoking abstinence. Following standardized audiotaped instructions, participants then smoked 3 high-nicotine (1.1-mg) cigarettes 25 minutes apart.<sup>19,26</sup>

After smoking each cigarette, the acute effects of smoking were measured on 4 dimensions (physical effects, cognitive effects, rush or high, and reward). The Nicotine Effects Scale was used to measure the experience of physical effects (ie, nausea, buzzing, dizziness, heart pounding, unpleasantness, and palms sweating), cognitive effects (ie, calm, attentive, satisfied, euphoric, energized, stimulated, clear headed, and mentally alert), and the extent to which participants experienced a rush or high. For each item, participants responded on a Likert-type scale from 1 ("not at all") to 10 ("extremely"). The participants were also asked about how rewarding they found each cigarette using 3 items ("how satisfying was the cigarette?" "how enjoyable was the cigarette?" and "how much did you like the cigarette?"; Cronbach  $\alpha = .90$ ) on a scale from 1 to 10. These methods are identical to those used in previous studies.<sup>19,26</sup>

## SMOKING CESSATION

We examined associations of variation in *CHRNA4* 3' UTR SNP *rs2236196* and 5' promoter SNP *rs6122429* with treatment outcome (smoking abstinence) in an open-label randomized clinical trial of transdermal nicotine patch vs nicotine nasal spray. The methods of this study are described briefly herein; they are described in detail elsewhere.<sup>27</sup>

The study included 353 current cigarette smokers of European ancestry who were aged 18 years or older and had smoked at least 10 cigarettes/d for the 12 months before study enrollment. Exclusion criteria included unstable angina, pregnancy or lactation, uncontrolled hypertension, current diagnosis of major depression, heart attack, or stroke within the past 6 months, current treatment or recent diagnosis of cancer, current diagnosis or history of a mania or psychosis, current diagnosis of drug or alcohol dependence (except for nicotine dependence), and current use of bupropion hydrochloride or nicotine-containing products other than cigarettes. Participants were recruited at Georgetown University, Washington, District of Columbia, and the University of Pennsylvania, Philadelphia, from February 15, 2000, to August 6, 2003.

## PROCEDURES

Participants responding to newspaper advertisements were screened for eligibility, completed a pretreatment assessment of demographics and smoking history, and then were randomized to receive 8 weeks of transdermal nicotine patch or nicotine nasal spray. All participants also received 7 sessions of standardized behavioral group counseling during 11 weeks; transdermal nicotine patch or nicotine nasal spray was initiated on the target quit date, which coincided with the third counseling session. Self-report data on smoking status were obtained at the end of treatment and at 6 months after the target quit date. Participants who reported complete smoking abstinence for the 7 days before the assessment were asked to complete an in-person visit for biochemical verification of smoking abstinence using the exhaled carbon monoxide reading. Of 165 participants self-reporting smoking abstinence at the end of treatment, 126 (76.4%) provided a carbon monoxide sample

for verification; of 103 participants reporting smoking abstinence at 6 months after the target quit date, 73 (70.9%) provided a carbon monoxide sample. At baseline, saliva cotinine level testing was performed using a gas-liquid chromatography method.<sup>28</sup>

## MEASURES

Genomic DNA samples were assayed as already outlined. Because of the small number ( $n=7$ ) of cases with 2 A alleles at *rs6122429*, we treated cases with 1 or 2 A alleles at that SNP as a single group for the purposes of the analysis. Similarly, because of the small number ( $n=23$ ) of cases with 2 C alleles at *rs2236196*, we treated cases with 1 or 2 C alleles at that SNP as a single group.

## Covariates

Sex, smoking rate, and serum cotinine level were assessed at baseline. Nicotine dependence was also assessed at baseline using the Fagerström Test for Nicotine Dependence.<sup>29</sup>

## Smoking Abstinence

In accord with recommendations of the Society for Research on Nicotine and Tobacco expert panel,<sup>30,31</sup> participants were classified as abstinent if they self-reported smoking abstinence (not even a puff) for each of the 7 days immediately before the follow-up point (ie, point prevalence) and provided a carbon monoxide reading of 10 ppm or lower. Consistent with these recommendations, we presumed that those who failed to complete the follow-up or failed to provide a biosample for biochemical verification had relapsed.

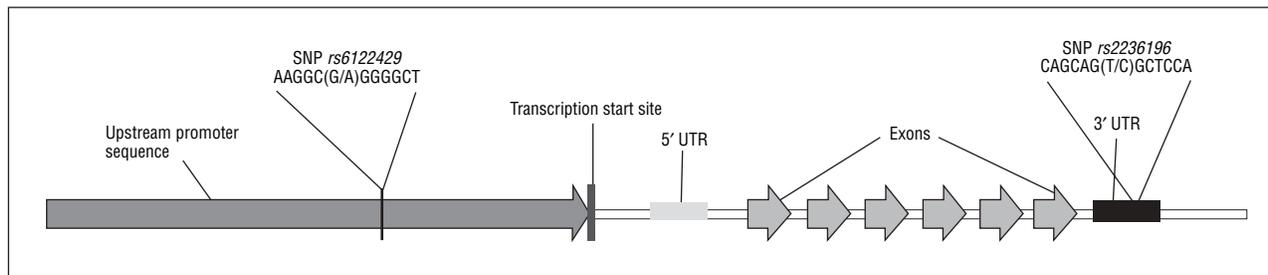
# RESULTS

## BIOINFORMATICS AND MOBILITY SHIFT ASSAY RESULTS

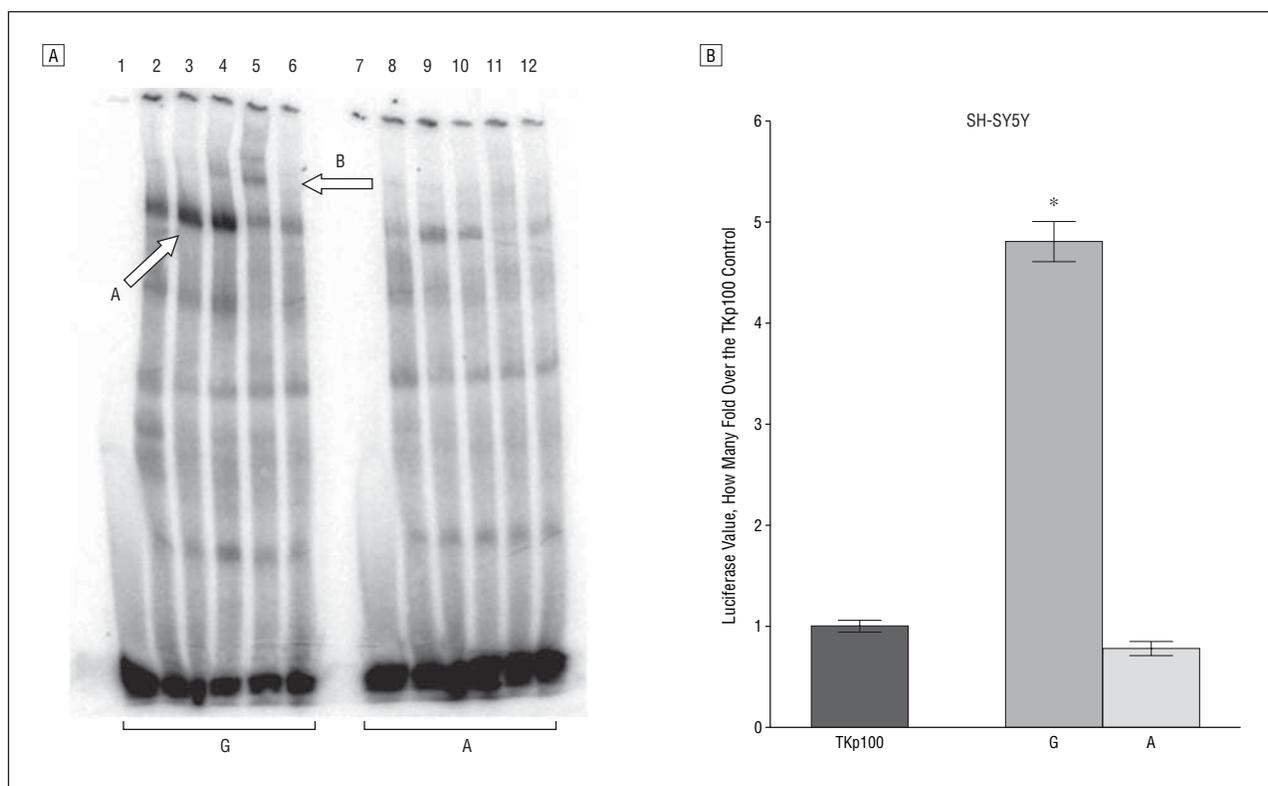
**Figure 1** shows the regulatory and coding regions of *CHRNA4* and the relative locations of the 2 SNPs (*rs6122429* and *rs2236196*). Linkage disequilibrium between the 2 SNPs was low at 0.319.

Initially, the 5' promoter and 3' UTR regions were examined using bioinformatics tools to identify SNPs that might alter binding sites in regulatory regions. Only the 5' promoter and 3' UTR regions were analyzed in this study because these are the regions that were most likely to harbor a regulatory SNP. Fifteen SNPs from the promoter region were screened using bioinformatics tools. Six were identified as SNPs that potentially changed a transcription factor binding site. To determine whether the 5' promoter SNPs altered transcription factor binding, reactions were completed using nuclear extracts for neuronal SH-SY5Y cells and labeled probes corresponding to each version of the SNP. Results of supershift analysis of the gels using antibodies to the respective transcription factors suggested that 1 SNP, *rs6122429*, altered an Sp1 binding site (**Figure 2A**).

In addition, analysis of the 3' UTR revealed that *rs2236196* putatively alters an mRNA binding site for the iron-responsive element (IRE). Nucleotide substitutions in this site lead to a substantial loss of binding af-



**Figure 1.** Schematic representation of the human *CHRNA4* gene showing the locations of the promoter, 5' untranslated region (UTR), coding exons, 3' UTR, and 2 single-nucleotide polymorphisms (SNPs) investigated in this study, 6122429 G/A and 2236196 T/C.



**Figure 2.** Results of biochemical assays for the *CHRNA4* single-nucleotide polymorphism (SNP) *rs6122429* analyzed in this study. A, Mobility shift assay using radioactive oligonucleotides containing the region flanking the *rs6122429* SNP with the G allele (lanes 1-6) or the A allele (lanes 7-12). Lane 1 indicates probe with no nuclear extract; lanes 2 and 8, probe plus HeLa cell nuclear extract; lanes 3 and 9, probe plus SH-SY5Y neuronal cell nuclear extract; lanes 4 and 10, probe plus SH-SY5Y nuclear extract and antilamin A/C nuclear extract (control); lanes 5 and 11, probe plus SH-SY5Y nuclear extract plus anti-Sp1 antibody; and lanes 6 and 12, probe plus SH-SY5Y nuclear extract and anti-Sp3 antibody. Binding activity is evident with the *rs6122429* G oligo (arrow A), which is greatly reduced in the *rs6122429* A oligo. This binding activity is supershifted by the antibody to Sp1 (arrow B) and is reduced by the anti-Sp3 antibody. B, Neuronal SH-SY5Y cells. The *rs6122429* A (rare) SNP sensor construct was approximately 3-fold to 5-fold less active than the *rs6122429* G (common) SNP sensor construct. Each bar represents the mean  $\pm$  SEM of 4 experiments, each with 4 to 8 wells. TKp100 indicates thymidine kinase promoter.

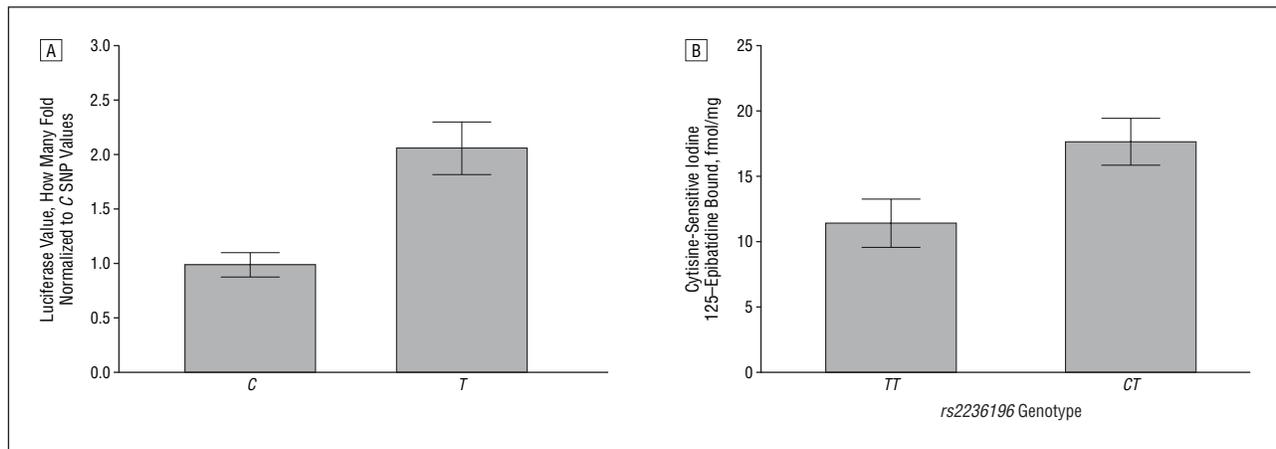
finity.<sup>32-34</sup> Recent findings suggest that the IREs also regulate translation of genes involved in CNS function.<sup>35</sup> In the 3' UTR mRNA for *CHRNA4*, *rs2236196* changes the fourth nucleotide in the consensus sequence for the binding site. If the IRE in the 3' UTR of *CHRNA4* plays a regulatory role, the T version of the SNP should be associated with an increase in mRNA levels via the stability provided by the IRE site, whereas the C version of the SNP would be associated with decreased levels of mRNA.

#### LUCIFERASE RESULTS

Given that *rs6122429* seemed to alter a binding site for Sp1, sensor constructs for each version of the SNP were

placed upstream of a minimal promoter driving a luciferase gene. Analyses of the sensor constructs already described indicated that expression was 5-fold greater using the G substitution for *rs6122429* in SH-SY5Y cells (Figure 2A and B).

Because the *rs2236196* SNP putatively altered the consensus sequence for an IRE in the 3' UTR mRNA, its effect on gene expression was also examined in a luciferase activity assay using reporter constructs containing the *CHRNA4* 3' UTR with different alleles of the SNP. Experiments in SH-SY5Y cells transfected with these constructs indicated that the construct with the T version was approximately 2-fold more active than the construct with the C version (Figure 3A).



**Figure 3.** Analysis results. A, Bar graph showing the how many fold difference in luciferase activity in neuronal SH-SY5Y cells between constructs containing the 3' untranslated region sequence of both alleles of *rs2236196*. The construct with the T version was approximately 2-fold more active than the construct with the C version. B, The binding levels in postmortem brain tissue for samples with the TC genotype vs the TT genotype for *rs2236196*. Binding was decreased among individual samples with the TT genotype for *rs2236196* in the nucleus accumbens. SNP indicates single-nucleotide polymorphism. Limit lines in A and B indicate standard error of the mean.

### α4β2 BINDING IN POSTMORTEM BRAIN TISSUE

For *rs6122429*, 17 individuals had a GG genotype, and 3 individuals had an AG genotype. Because of the lack of variability for *rs6122429*, this SNP was not examined further. For *rs2236196*, 10 individuals had a TT genotype, and 10 individuals had a TC genotype. The α4β2 binding was quantified in the nucleus accumbens, and the association between *rs2236196* and binding levels was assessed. An analysis of covariance with smoking status as a covariate revealed that individuals with the TC genotype for *rs2236196* demonstrated statistically significantly greater α4β2 binding in the nucleus accumbens than individuals with the TT genotype ( $P=.02$ ) (Figure 3B).

### EFFECT OF THE SNPs ON BEHAVIORAL SENSITIVITY TO NICOTINE

Of 316 participants, 54.0% were male. With respect to ancestry, the breakdown was 80% European, 2% African, 3% Asian, 9% Hispanic, and 6% Native American. The mean±SD age of the participants was 26.9±9.0 years. The participants smoked a mean±SD of 16.0±7.8 cigarettes/d at baseline. The mean±SD score on the Fagerström Test for Nicotine Dependence was 4.0±2.2. For *rs2236196*, 49% of the sample had a TT genotype, 41% had a TC genotype, and 10% had a CC genotype. The frequency of the C allele was 30%, while the frequency of the T allele was 70%. These frequencies are consistent with the National Center for Biotechnology Information database, which reports that the C allele frequency is 31% in individuals of European ancestry (66% for those of African ancestry and 8% for those of Chinese ancestry). For *rs6122429*, 64% of the sample had a GG genotype, 33% had an AG genotype, and 3% had an AA genotype. The frequency of the A allele was 19%, while the frequency of the G allele was 81%. The genotypes for *rs2236196* and *rs6122429* were in Hardy-Weinberg equilibrium (Pearson product moment correlation  $\chi^2=0.13$ ,

$P>.10$  and  $\chi^2=0.30$ ,  $P>.10$ , respectively). Genotype groups did not differ on any of the variables already described ( $P>.05$ ). Given the low number of individuals with the infrequent AA genotype for *rs6122429* and CC genotype for *rs2236196*, these individuals were grouped with the heterozygotes in the main analyses. This decision was necessary because the sample size for the groups that are homozygous for the rare alleles is so small that the standard error of the mean is much larger for this group and statistical power is compromised.

The main statistical analysis involved estimating a series of random-coefficient regression models<sup>36</sup> to test the influence of the genetic variations on responses to nicotine. The *rs2236196* and *rs6122429* were the 2 SNPs that were advanced at the more basic levels, and they were the only 2 SNPs analyzed in the behavioral data. In these models, responses to nicotine are regressed onto the random effects of trial and the intercept and the fixed effects of trial (ie, the SNP, cigarette, and SNP × cigarette interaction). The analyses were focused on sensitivity to the effects of nicotine, which was measured using a nicotine effects questionnaire that assesses sensitivity on the following 3 dimensions: the experience of physiological effects, the experience of a rush or high, and the experience of cognitive effects. In addition, we examined the self-reported rewarding effects of smoking. These models were estimated using commercially available statistical software (PROC MIXED procedure; SAS version 9.1, SAS Institute, Cary, North Carolina).<sup>36-38</sup> Random-coefficient regression models within the PROC MIXED procedure allow for the specification of repeated observations across time within individuals, as well as the clustered nature of a subset of the subjects who were siblings.

The analyses revealed that *rs2236196* was associated with the subjective physiological effects of smoking (eg, heart pounding and dizziness) and the experience of a rush or high after each of the 3 cigarettes (**Table 1**). Individuals with the TC genotype demonstrated greater subjective sensitivity on these dimensions after smoking each of the cigarettes compared with individuals

with the *TT* genotype (Figure 4A and B). The analyses also revealed a statistically significant SNP × cigarette interaction on sensitivity to the cognitive effects of nicotine, such that individuals with the *TC* genotype reported feeling more of the cognitive effects of smoking (eg, alert and attentive) than individuals with the *TT* genotype after the first cigarette, although there was no difference after the second and third cigarettes (Figure 4C). With respect to *rs6122429*, there was a statistically significant SNP × cigarette interaction, such that individuals with the *GG* genotype reported greater reward after the third cigarette but not after the first or second cigarette (Figure 4D).

To further ensure that the findings were not due to an artifact given the inclusion of the siblings, 1 sibling per sibling pair was randomly deleted. The analyses were repeated using the PROC MIXED procedure and were then repeated using the more common analysis of variance. All of the effects that were found to be statistically significant in the original analysis remained statistically significant in the subsequent analyses.

### TREATMENT OUTCOME ANALYSES

*t* Tests were used to assess bivariate associations of genotype with baseline smoking rate, nicotine dependence, and serum cotinine level;  $\chi^2$  tests were used to assess associations of genotype with sex, treatment assignment, and smoking abstinence. A longitudinal logistic regression analysis of smoking abstinence at the end of treatment and at 6 months after the target quit date was performed using the general effect estimation procedure (XTGEE) (STATA, StataCorp LP, College Station, Texas). Predictors included in the model were sex, the *rs6122429* genotype, the *rs2236196* genotype, nicotine dependence, treatment (transdermal nicotine patch or nicotine nasal spray), time point (end of treatment or 6 months after the target quit date), and all possible 2-way interactions among the *rs6122429* genotype, the *rs2236196* genotype, treatment condition, and time point. In addition, the following 3-way interactions were examined: the *rs6122429* genotype × treatment condition × time point and the *rs2236196* genotype × treatment condition × time point. All predictors were initially entered as a block, after which statistically nonsignificant ( $P \geq .10$ ) interaction terms were allowed to drop out (except for lower-order components of statistically significant higher-order interactions).

### Descriptive Data

Of 353 eligible participants, 190 (53.8%) were male. Participants smoked a mean ± SD of 23.5 ± 9.2 cigarettes/d at baseline. The mean ± SD score on the Fagerström Test for Nicotine Dependence was 5.53 ± 2.24, and the mean ± SD cotinine level was 249.9 ± 121.6 µg/L (to convert to nanomoles per liter, multiply by 5.675). For *rs6122429*, 2.0% of the cases were of *AA* genotype, 23.8% were of *AG* genotype, and 74.2% were of *GG* genotype. The *A* allele frequency was 13.9%. For *rs2236196*, 53.8% of the cases were of *TT* genotype, 39.7% were of *TC* genotype, and 6.5% were of *CC* genotype. The *C* allele frequency was 26.3%.

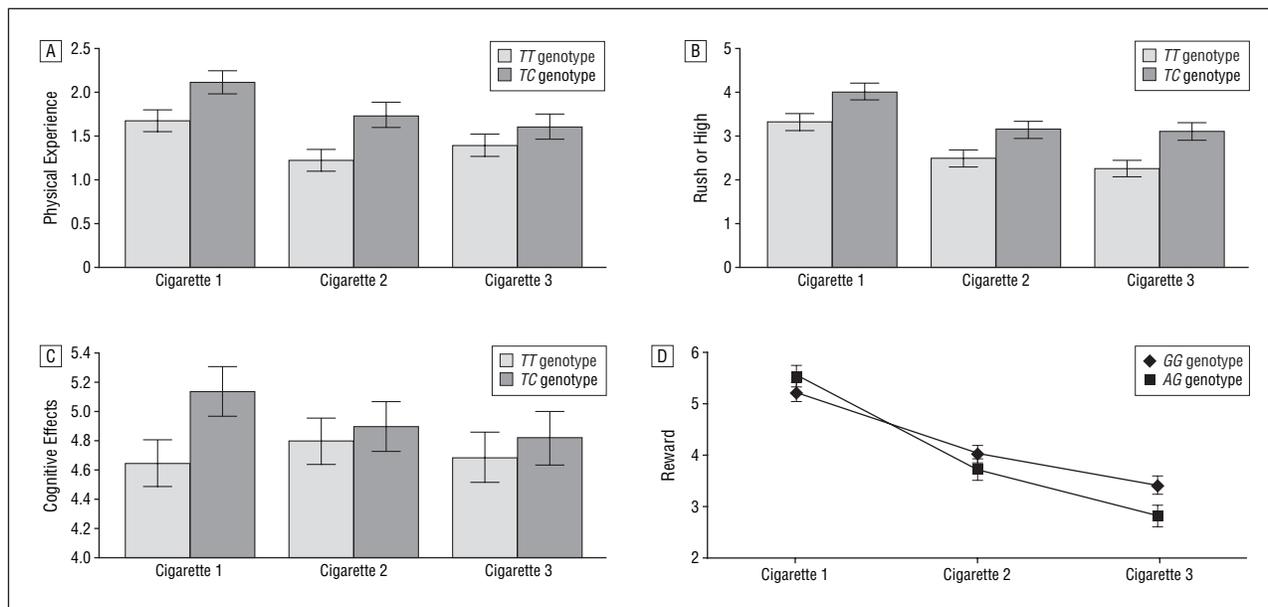
**Table 1. Parameter Estimates for the Fixed Effects of Single-Nucleotide Polymorphism (SNP) (*rs2236196* and *rs6122429*), Cigarette (Cigarettes 1-3), and SNP × Cigarette Interaction on Acute Responses to Smoking After 8 Hours of Smoking Abstinence**

Effect	Parameter Estimate	SE	<i>t</i> Test	<i>P</i> Value
<b><i>rs2236196</i></b>				
Physiological				
SNP	0.53	0.18	2.93	.003
Cigarette	-0.15	0.06	-2.51	.01
SNP × cigarette	-0.11	0.08	-1.25	.26
Rush or high				
SNP	0.64	0.26	2.43	.02
Cigarette	-0.54	0.07	-7.14	.001
SNP × cigarette	0.09	0.100	0.84	.41
Cognitive				
SNP	0.40	0.24	1.69	.08
Cigarette	0.02	0.06	0.30	.72
SNP × cigarette	-0.16	0.08	-1.94	.03
Reward				
SNP	-0.03	0.26	-0.11	.76
Cigarette	-1.07	0.10	-10.74	.001
SNP × cigarette	-0.02	0.14	-0.12	.98
<b><i>rs6122429</i></b>				
Physiological				
SNP	0.23	0.19	1.23	.20
Cigarette	-0.25	0.05	-4.85	.001
SNP × cigarette	0.10	0.09	1.16	.25
Rush or high				
SNP	0.55	0.28	1.99	.05
Cigarette	-0.49	0.06	-7.51	.001
SNP × cigarette	-0.07	0.11	-0.63	.63
Cognitive				
SNP	0.14	0.25	0.56	.61
Cigarette	-0.06	0.05	-1.16	.16
SNP × cigarette	-0.05	0.09	-0.57	.75
Reward				
SNP	0.28	0.28	1.02	.39
Cigarette	-0.92	0.08	-11.37	.001
SNP × cigarette	-0.46	0.14	-3.27	.002

The genotypes for *rs6122429* were in Hardy-Weinberg equilibrium ( $\chi^2=0.01$ ,  $P=.93$ ), as were the genotypes for *rs2236196* ( $\chi^2=0.17$ ,  $P=.68$ ). Cases with *TC* or *CC* genotype at *rs2236196* were marginally more likely to be female than cases with *TT* genotype (58.9% vs 49.5%;  $\chi^2=3.13$ ,  $P=.08$ ); *rs6122429* genotype was not statistically significantly associated with sex ( $P=.14$ ).

### Genotype Associations With Pretreatment Smoking Practices

Cases with *AA* or *AG* genotype at *rs6122429* had statistically significantly lower baseline scores on the Fagerström Test for Nicotine Dependence than cases with *GG* genotype (mean ± SD score, 5.07 ± 2.17 for *AA* or *AG* genotype and 5.69 ± 2.24 for *GG* genotype;  $t_{351}=2.30$ ,  $P=.02$ ). They also smoked statistically significantly fewer cigarettes per day at baseline (mean ± SD, 21.7 ± 8.8 for *AA* or *AG* genotype and 24.1 ± 9.3 for *GG* genotype;  $t_{350}=2.19$ ,  $P=.03$ ) and had marginally lower cotinine levels (mean ± SD, 231.2 ± 101.4 µg/L for *AA* or *AG* genotype and 256.4 ± 127.3 µg/L for *GG* genotype;  $t_{351}=1.71$ ,  $P=.09$ ).



**Figure 4.** Individuals with the *TC* genotype at *rs2236196* demonstrated statistically significantly ( $P = .003$  and  $P = .02$ , respectively) more sensitivity to nicotine in terms of the physical experience and rush or high across the 3 cigarettes (A and B). Significant interaction terms suggested that individuals with the *TC* genotype were more sensitive to the cognitive effects of nicotine after the first cigarette ( $P = .03$ ) (C) and that individuals with the *GG* genotype at *rs6122429* reported greater reward after the third cigarette ( $P = .002$ ) (D). Limit lines in A, B, and C indicate standard error of the mean.

**Table 2. Final Longitudinal Logistic Regression Model of Smoking Abstinence at the End of Treatment and at 6 Months After the Target Quit Date Among 353 Subjects<sup>a</sup>**

Predictor	Odds Ratio (95% Confidence Interval)	P Value
Sex [female = 1]	0.66 (0.43-1.03)	.07
Nicotine dependence	0.91 (0.83-1.00)	.06
Time point [6 mo after the target quit date = 1]	0.48 (0.30-0.78)	.003
Treatment condition [nicotine nasal spray = 1]	0.83 (0.45-1.53)	.54
<i>rs2236196</i> Genotype [ <i>C</i> * genotype = 1] <sup>b</sup>	1.05 (0.54-2.03)	.90
<i>rs6122429</i> Genotype [ <i>GG</i> genotype = 1]	0.93 (0.56-1.52)	.76
<i>rs2236196</i> Genotype × treatment condition	0.89 (0.36-2.20)	.80
<i>rs2236196</i> Genotype × time point	0.54 (0.25-1.17)	.12
Treatment condition × time point	0.74 (0.36-1.50)	.40
<i>rs2236196</i> Genotype × treatment condition × time point	3.87 (1.36-11.01)	.01

<sup>a</sup>The initial model also included the following interaction terms: *rs6122429* genotype × treatment condition, *rs6122429* genotype × time point, and *rs6122429* genotype × treatment condition × time point. All were statistically nonsignificant ( $P \geq .10$ ) and dropped out.

<sup>b</sup>*C*\* genotype indicates cases with 1 or 2 *C* alleles at *rs2236196*.

The *rs2236196* genotype was not statistically significantly associated with these pretreatment variables ( $P \geq .11$ ).

### Treatment Assignment

One hundred sixty-five participants (46.7%) were assigned to the transdermal nicotine patch condition, and

188 participants (53.3%) were assigned to the nicotine nasal spray condition. There were no statistically significant differences between the treatment groups in sex, serum cotinine level, nicotine dependence, number of cigarettes smoked per day, or the *rs6122429* or *rs2236196* genotype ( $P \geq .12$ ).

### Smoking Abstinence

Results of the final longitudinal logistic regression analysis of smoking abstinence at the end of treatment and at 6 months after the target quit date are summarized in **Table 2**. There was a statistically significant 3-way interaction of *rs2236196* genotype × treatment condition × time point ( $P = .01$ ). To compare the loss of smoking abstinence during the follow-up period across 4 subgroups, separate longitudinal logistic regression analyses of smoking abstinence were performed for each subgroup, using sex, time point, nicotine dependence, and the *rs6122429* genotype as predictors. Among 90 individuals with *TC* or *CC* genotype who received nicotine nasal spray, the effect of time point was statistically nonsignificant (odds ratio, 0.75; 95% confidence interval, 0.47-1.19;  $P = .22$ ), indicating no statistically significant reduction in smoking abstinence in that subgroup as a whole. By contrast, in each of the other 3 subgroups (range, 73-98 subjects), the effect of time point was statistically significant (odds ratio range, 0.25-0.48;  $P$  value range,  $P = .002$  to  $P < .001$ ).

### POPULATION STRATIFICATION

For population stratification to have confounded the analyses in the present study, allele frequencies for *rs6122429* and *rs2236196* must differ across groups with different ancestries. To address the issue of population

stratification, we first assessed whether allele frequencies were different among the ancestral groups in our study.<sup>39-41</sup> In our sample, the allele frequencies for *rs2236196* differed between those of European and Native American and African ancestry. The latter 2 groups constituted a small part of the sample, making it unlikely that population stratification might explain the results. Nonetheless, the issue was addressed in the analyses by repeating all of the analyses of the behavioral data using ancestry as a covariate and by repeating the analyses without the individuals of Native American and African ancestry. All of the results of the analyses remained statistically significant.

## COMMENT

The present investigation used an interdisciplinary translational approach spanning multiple levels of analysis (including bioinformatics, cell culture models, protein in postmortem brain tissue, human laboratory models, and analyses of treatment outcome data) to examine the influence of *CHRNA4* on nicotine dependence. A translational approach that emphasizes basic and applied approaches has a distinct advantage over treatment studies that lack basic science or basic science studies that lack a clinical translation. Analyses of mRNA and protein levels in postmortem brain tissue are critical for demonstrating that an SNP may be functional at a basic biological level. Analyses at the behavioral and clinical levels are important in terms of demonstrating that the biological effect of a locus penetrates these levels of analysis. Investigations that include multiple levels of analysis will provide important information regarding how genetic variation may affect the cause and treatment of addictive behavior.

In the present study, transfection investigations using luciferase reporter constructs revealed a statistically significant effect of the 2 SNPs in question on gene expression. A sensor construct containing the G version of the 5' promoter SNP *rs6122429* showed greater luciferase activity in neuronal cells than a construct containing the A version of this SNP (Figure 2B). Results of bioinformatics and DNA binding assays suggest that this difference is due to a difference in the ability of this region to bind to the transcriptional activator Sp1. In addition, constructs containing the *CHRNA4* 3' UTR with the T version of SNP *rs2236196* showed statistically significantly greater luciferase activity in SH-SY5Y cells than constructs containing the C version (Figure 3A). Because the objective of the present study was to identify functional SNPs for further analyses in behavioral assays and not to definitively establish the molecular mechanism underlying the effects of this SNP on gene expression, the biological pathways involved in the difference in luciferase activity caused by this 3' UTR SNP have not yet been identified. Future studies can address this hypothesis on a molecular level.

The  $\alpha 4\beta 2$  receptor assays indicated greater binding among individuals with the TC genotype. Furthermore, *rs2236196* was associated with changes in sensitivity to the acute effects of nicotine in a human laboratory model

and with response to nicotine therapy in a sample of treatment-seeking individuals. Specifically, individuals with the TC genotype at *rs2236196* reported statistically significantly greater sensitivity to the acute effects of smoking after 8 hours of smoking abstinence, including a statistically significantly greater rush or high after each of the 3 cigarettes than individuals with the TT genotype. Finally, *rs2236196* was associated with differential treatment response in a trial comparing different forms of nicotine therapy. It should be noted that 2 previous studies<sup>8,9</sup> that examined nicotine dependence phenotypes also noted a statistically significant, or close to significant, association with *rs2236196*.

The obvious, and most parsimonious, explanation for these findings is that *rs2236196* leads to a change at the protein level, which in turn leads to a change in CNS sensitivity to cholinergic stimulation. The behavioral finding that individuals with the TC genotype experience a greater rush or high, increased subjective physiological effects, and more cognitive effects after acute administration is consistent with this explanation. It also stands to reason that individuals who have a greater sensitivity to the acute effects of smoking, including a greater rush or high, might respond differently to a form of nicotine therapy that reaches the CNS quickly and produces acute effects (ie, nicotine nasal spray),<sup>42</sup> as opposed to a form of delivery that involves slow absorption and minimal acute effects (ie, transdermal nicotine patch). Not surprisingly, individuals with the TC genotype, which was associated with increased  $\alpha 4\beta 2$  binding and greater sensitivity to the acute effects of smoking, were more likely to maintain treatment gains during the follow-up period when they received nicotine nasal spray. Therefore, for individuals with the TC genotype, forms of nicotine replacement that reach the CNS more quickly (eg, nicotine gum, nasal spray, and inhaler lozenge) may more closely mimic their experience of cigarette smoking and be more useful as a cessation aid than transdermal nicotine patch. Although the statistically significant *rs2236196* genotype  $\times$  treatment condition  $\times$  time point interaction suggests that *rs2236196* may be an important moderator of outcomes, analyses of this marker in independent clinical trials of nicotine therapy with a no-treatment control group will help to clarify whether this variant plays a role in overall liability to relapse or in response to pharmacotherapy.

**Submitted for Publication:** September 1, 2006; final revision received January 31, 2007; accepted February 4, 2007.

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**Financial Disclosure:** Dr Benowitz is a consultant to several pharmaceutical companies that market smoking cessation products.

**Funding/Support:** This study was supported by grants DA14642 and AA012238 from the National Institute on Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism (Dr Hutchison), by grant K01AR050505 from the National Institute of Arthritis and Musculo-

skeletal and Skin Diseases (Dr Allen), by grant P5084718 from the National Cancer Institute and the National Institute on Drug Abuse (Dr Lerman), and by grant DA14369 from the National Institute on Drug Abuse (Dr Stitzel). Tissues were received from the New South Wales Tissue Resource Centre or the Prince of Wales Medical Research Institute Tissue Resource Centre, which are supported by the National Health and Medical Research Council of Australia, The University of Sydney, the Prince of Wales Medical Research Institute, the Neuroscience Institute of Schizophrenia and Allied Disorders, the National Institute of Alcohol Abuse and Alcoholism, and the New South Wales Department of Health.

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