

# A *CHRNA5* Smoking Risk Variant Decreases the Aversive Effects of Nicotine in Humans

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Genome-wide association studies have implicated the *CHRNA5-CHRNA3-CHRNA4* gene cluster in risk for heavy smoking and several smoking-related disorders. The heavy smoking risk allele might reduce the aversive effects of nicotine, but this hypothesis has not been tested in humans. We evaluated the effects of a candidate causal variant in *CHRNA5*, rs16969968, on the acute response to nicotine in European American (EA) and African American (AA) smokers ( $n=192$ ; 50% AA; 73% male). Following overnight abstinence from nicotine, participants completed a protocol that included an intravenous (IV) dose of saline and two escalating IV doses of nicotine. The outcomes evaluated were the aversive, pleasurable, and stimulatory ratings of nicotine's effects, cardiovascular reactivity to nicotine, withdrawal severity, and cognitive performance before and after the nicotine administration session. The heavy smoking risk allele (rs16969968\*A; frequency = 28% (EA) and 6% (AA)) was associated with lower ratings of aversive effects ( $P < 5 \times 10^{-8}$ ) with marked specificity. This effect was evident in EA and AA subjects analyzed as separate groups and was most robust at the highest nicotine dose. Rs16969968\*A was also associated with greater improvement on a measure of cognitive control (Stroop Task) following nicotine administration. These findings support differential aversive response to nicotine as one likely mechanism for the association of *CHRNA5-CHRNA3-CHRNA4* with heavy smoking.

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## INTRODUCTION

Nicotine dependence (ND) is a multifactorial disorder associated with high rates of morbidity and mortality. The estimated heritability for ND is about 0.59 in men and 0.46 in women (Li *et al*, 2003), indicating significant genetic contributions. Studies on the genetics of ND have provided important insight into the molecular mechanisms underlying ND and several associated disorders, such as heavy smoking and lung cancer. Variants encoded within the *CHRNA5-CHRNA3-CHRNA4* gene cluster on chromosome 15 are strong candidates for ND risk, the severity of ND measured by Fagerström Test for Nicotine Dependence scores, the number of cigarettes smoked per day, as well as the risk for lung cancer, chronic obstructive pulmonary disease, and peripheral vascular disease (Amos *et al*, 2008; Berrettini *et al*, 2008; Bierut *et al*, 2007; Bierut *et al*, 2008; Chen *et al*, 2012; Chen *et al*, 2009; Gelernter *et al*, 2015; Hansen *et al*, 2010; Hung *et al*, 2008; Li *et al*, 2010; Lips *et al*, 2010; Liu *et al*, 2010; Saccone *et al*, 2010; Saccone *et al*, 2007; Sarginson *et al*,

2011; Sherva *et al*, 2010; Spitz *et al*, 2008; Thorgeirsson *et al*, 2008; Tobacco and Genetics Consortium, 2010; Wang *et al*, 2009).

A highly conserved non-synonymous SNP, rs16969968 (G > A; amino acid 398 D > N), in the *CHRNA5* gene, which encodes the  $\alpha 5$  subunit of nicotinic acetylcholine receptors (nAChR), apparently mediates some of the risk effects associated with the *CHRNA5-CHRNA3-CHRNA4* gene cluster (Saccone *et al*, 2007). This hypothesis is supported by large GWAS meta-analyses and *in vitro* studies that have characterized the function of 398 D > N and behavioral studies of  $\alpha 5$  in animal models (Bailey *et al*, 2010; Bierut *et al*, 2008; Fowler *et al*, 2011; Frahm *et al*, 2011; Jackson *et al*, 2010; Kuryatov *et al*, 2011; Saccone *et al*, 2010; Tammimaki *et al*, 2012; Tobacco and Genetics Consortium, 2010). The *CHRNA5* risk allele, rs16969968\*A (398N), encodes a protein that forms nACh receptors with lower activity in several *in vitro* paradigms (Bierut *et al*, 2008; Kuryatov *et al*, 2011). In a study with the ( $\alpha 4\beta 2$ ) $\alpha 5$  nAChR-transfected HEK293 cells, nAChR subunits containing the  $\alpha 5$  risk allele (398N) had twofold lower maximal response when challenged with epibatidine, a nicotinic agonist, compared with the ( $\alpha 4\beta 2$ ) $\alpha 5$  nAChRs that contained the  $\alpha 5$  protective allele (398D) (Bierut *et al*, 2008). Further, when the risk allele of  $\alpha 5$  was incorporated into ( $\alpha 4\beta 2$ )\* nAChRs expressed in *Xenopus* oocytes, there was an increase in short-term desensitization

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and lower calcium permeability relative to ( $\alpha 4\beta 2$ )\* nAChRs that contained the protective allele (Kuryatov *et al*, 2011). These functional differences for 398 D>N were observed for ( $\alpha 4\beta 2$ ) $\alpha 5$ , the  $\alpha 5$  nAChR subtype that is most common in the brain, and not observed for  $\alpha 5$  nAChR subtypes that are rare in the brain but common in the periphery (Kuryatov *et al*, 2011).

The function of  $\alpha 5$  as evaluated in animal models is consistent with and relevant to nicotine's cognitive-enhancing and subjective drug effects. Under highly demanding conditions, knockout  $\alpha 5$  mice have reduced cognitive performance in attention tasks compared with the wild-type mice; yet under these demanding conditions, nicotine negatively affected the performance of wild-type mice but not  $\alpha 5$  knockouts (Bailey *et al*, 2010). At high doses of nicotine but not at low doses, mice lacking the  $\alpha 5$  subunit have reduced aversive and elevated reward responses to nicotine relative to wild-type animals (Jackson *et al*, 2010). Although the  $\alpha 5$  subunit is expressed widely in the central and peripheral nervous system as part of various nAChRs, including  $\alpha 4\beta 2$ \*,  $\alpha 3\beta 2$ \*, or  $\alpha 3\beta 4$ \*, rodent studies suggest that  $\alpha 5$  expressed in the medial habenula may be most relevant to the risk effects marked by rs16969968 (Fowler *et al*, 2011; Frahm *et al*, 2011). Fowler *et al* (2011) showed that viral-mediated expression of  $\alpha 5$  specifically in the medial habenula of  $\alpha 5$  knockouts is sufficient to restore the aversive effects of nicotine to wild-type levels. In contrast, RNAi-mediated knockdown of  $\alpha 5$  specifically in habenulo-interpeduncular tract cells of wild-type animals induced a nicotine-aversion phenotype that was similar to the constitutive  $\alpha 5$  knockouts. These important studies highlight the critical role of  $\alpha 5$  in regulating the aversive effects of nicotine. Based on these studies, it has been inferred that the low activity form of  $\alpha 5$ , encoded by rs16969968\*A, renders individuals less sensitive to the aversive effect of nicotine. Although an intriguing mechanism to explain the risk associated with rs16969968\*A, there is limited direct evidence from human studies to support this type of specific behavioral effect of rs16969968 on aversion to nicotine or the cognitive-enhancing effects of nicotine.

Characterizing the behavioral effects of rs16969968 in humans could lead to significant health benefits and increase our fundamental understanding of a well-established behavioral genetics effect. This could be established by measuring subjective and objective responses to nicotine in humans in a laboratory setting, but cigarette smoking is a complex behavior and the amount of nicotine delivered via smoking can be highly variable (Hatsukami *et al*, 2006; Hoffmann and Wynder, 1986). Assessing the relevant properties of rs16969968 in humans requires a non-variable nicotine delivery system. Among pure nicotine delivery systems, delivery via patch is slow and does not produce subjective rewarding effects. Faster nicotine delivery systems, including nicotine gum and nasal spray, produce minimal or no drug 'liking' effects and are not preferred over placebo by smokers in self-administration procedures (Perkins, 1996; Schneider *et al*, 2005; Schuh *et al*, 1997). We have developed an intravenous (IV) nicotine administration paradigm to evaluate accurately and precisely responses to nicotine that are relevant to smoking behavior (Sofuoglu *et al*, 2003; Sofuoglu *et al*, 2005; Sofuoglu *et al*, 2006). The advantages of this IV paradigm are precise nicotine dosing and a rapid nicotine

delivery that is comparable to the bolus effect of smoking. This IV nicotine paradigm produces robust 'pleasurable', 'stimulatory', and 'aversive' subjective effects that are similar to cigarette smoking. Unlike other pure nicotine delivery systems, this paradigm is reinforcing and is self-administered by male and female smokers (Sofuoglu *et al*, 2008). This paradigm also, similar to cigarette smoking, attenuates symptoms of nicotine withdrawal and craving to smoke and improves cognitive performance in abstinent smokers (DeVito *et al*, 2013; Herman *et al*, 2013; Sofuoglu *et al*, 2012).

Here we used this laboratory paradigm to evaluate the effects of rs16969968 in European American (EA;  $N=96$ ) and African American (AA;  $N=96$ ) smokers. The dose-dependent response to nicotine was evaluated for multiple outcomes, including the subjective ratings of 'aversive', 'pleasurable', and 'stimulatory' effects. Cardiovascular response to nicotine, symptoms of nicotine withdrawal, and effects on cognitive performance were also evaluated.

## MATERIALS AND METHODS

### Subjects and Genotyping

One hundred and ninety-two non-treatment-seeking EA (50%) and AA (50%) smokers were recruited from the New Haven, Connecticut area for a nicotine laboratory study. All provided written informed consent before participating in the study, and subjects were paid for their participation. Institutional review boards at Yale University and the VA Connecticut Healthcare System approved the study. Seventy-three percent of subjects were male, and the mean age was 36.3 (SD=8.9) years. Some subjects from this population have been included in previous studies (DeVito *et al*, 2013; Herman *et al*, 2013; Jensen *et al*, 2014; Sofuoglu *et al*, 2012). All subjects reported smoking 10–25 cigarettes/day for the past year, had expired carbon monoxide levels of  $\geq 10$  parts per million at initial screening, and had no major medical problems as determined by a self-report, a physician's health check, and laboratory testing. Subjects were evaluated with the Structured Clinical Interview for DSM-IV (First *et al*, 1996) and subjects who met criteria for a current psychiatric disorder, including dependence on alcohol or drugs (other than nicotine), were excluded from the study. A urine drug screen confirmed abstinence from drugs other than nicotine. Individuals on psychotropic medication or who were pregnant or breastfeeding were also excluded. DNA was extracted from whole blood using a commercial kit (PureGene; Gentra, Minneapolis, MN, USA). Subjects were genotyped for *CHRNA5* rs16969968 with a 2- $\mu$ l TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). The subjects were assigned to EA or AA group based on a previously described genetic marker method (Herman *et al*, 2013; Yang *et al*, 2005) or based on self-report.

### Laboratory Procedure and Assessments

Experimental sessions started at 0800 hours following an overnight abstinence from smoking, confirmed by expired carbon monoxide levels  $< 10$  parts per million and plasma nicotine levels  $< 4$  ng/ml. The study procedure has been described in detail elsewhere (Herman *et al*, 2013; Sofuoglu *et al*, 2012) At baseline, after IV lines were established,

subjects completed the Minnesota Nicotine Withdrawal Scale (MNWS) (Hughes and Hatsukami, 1986), the Brief Questionnaire of Smoking Urges (BQSU) (Tiffany and Drobes, 1991), and the Positive and Negative Affect Schedule (PANAS) (Watson *et al*, 1988). Subjects were then administered an IV dose of saline, followed by two escalating doses of nicotine (0.5 mg per 70 kg of body weight and 1 mg per 70 kg of body weight) in uniform order to avoid nicotine carryover into the saline dose. Nicotine was infused over 30 s IV via a forearm catheter. Each infusion was separated by 30 min. During the experimental session, the subjective effects of nicotine (and saline placebo) were assessed with the Drug Effects Questionnaire (DEQ) and blood pressure (systolic and diastolic) and heart rate (HR) were monitored. Each individual DEQ response was rated on a visual 100-mm scale that was then converted to a rating from 1 to 10. Approximately 20 min after the final dose of nicotine, subjects repeated the MNWS, BQSU, and PANAS. Two subjects (of 192) did not complete the MNWS and 6 did not complete the BQSU and PANAS.

The Stroop Task, The Running Memory Continuous Performance Task (CPT) and The Mathematical Processing Task (MPT) were performed at baseline and at the end of the session. This computer-based cognitive battery from Automated Neuropsychological Assessment Metrics (Reeves *et al*, 2007) has been described previously in detail for this paradigm (DeVito *et al*, 2013). One hundred and eighty-four subjects completed the cognitive assessments. The outcome analyzed for each cognitive task was the throughput score, a summary measure computed from the number of correct responses within the allotted time.

Plasma nicotine, cotinine, and 3'-hydroxycotinine levels prior to nicotine infusion were determined using HPLC interfaced with tandem mass spectrometry (LC/MS/MS) with deuterium-labeled internal standards, as previously described (Dempsey *et al*, 2004; Sofuoglu *et al*, 2012).

## Data Analysis

Data were analyzed with linear mixed models using the JMP Pro (v10.0.0) software (SAS Institute). The DEQ and cardiac response models included as independent variables the following fixed effects: rs16969968 (grouped as A carrier *vs* GG), age, sex, race (EA or AA), IV dose (saline, 0.5 mg nicotine and 1 mg nicotine per 70 kg body weight), minute post-IV infusion, the interaction of dose with rs16969968, the interaction of minute post-IV infusion with rs16969968, the interaction of minute post-IV infusion with dose, and the interaction of minute post-IV infusion with dose and rs16969968. Subject was included in the linear mixed model as a random effect. Additive genetic models were also used as indicated in the Results section, with copies of the minor allele coded as 0, 1, and 2 for GG, AG, and AA genotype groups, respectively. Based on prior work indicating that the responses to nine DEQ items were correlated, we clustered nine responses into three domains representing aversive, pleasurable, and stimulatory effects (Morean *et al*, 2013). The average response for 'feel anxious', 'feel down', and 'feel bad' formed the aversive domain; the average response for 'like', 'feel good', and 'want more' formed the pleasurable domain; and the average response for 'feel stimulated', 'feel effects',

and 'feel high' formed the stimulatory domain. The change in response to nicotine relative to saline for each drug effect domain (shown in Figure 1) was calculated by subtracting the value of the saline condition at 1, 3, 5, 8, and 10 min postinfusion from the corresponding value during the nicotine conditions for each individual, and the mean value of the five time points at each nicotine dose is reported. MNWS, PANAS, BQSU, ST, CPT, and MPT analysis models included the following fixed effects: rs16969968 (grouped as A carrier *vs* GG), age, sex, race (EA or AA), time point (baseline *vs* end of session), and the interaction of time point with rs16969968, and included subject as a random effect. Additional covariates included in *post-hoc* analyses are described in the text.

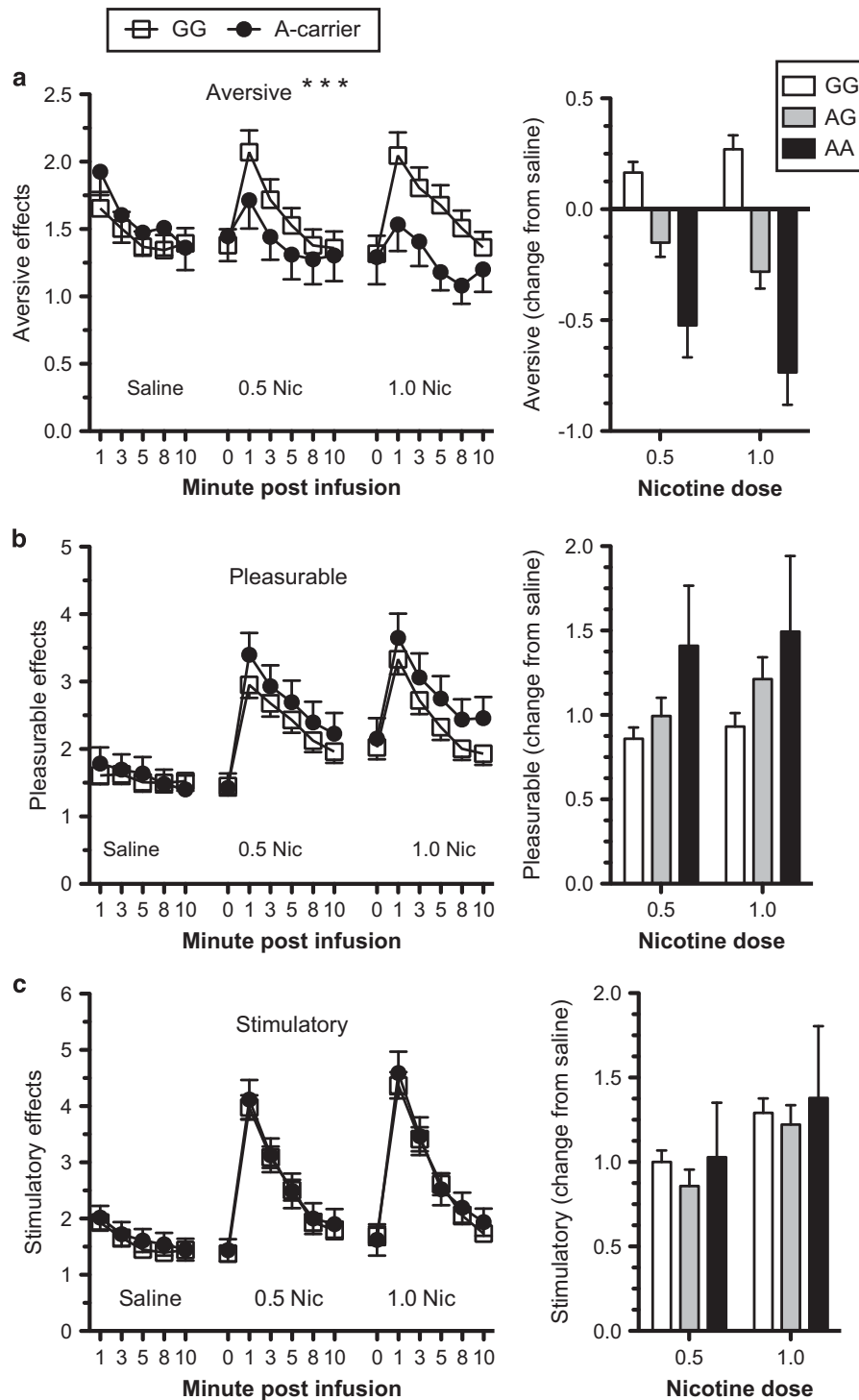
## RESULTS

### Demographic and Smoking-Related Characteristics

Rs16969968 genotypes for EA and AA subjects are shown in Table 1. The genotype counts for the EA and AA samples did not deviate from Hardy-Weinberg expectations. Smoking-related characteristics for the EA and AA subjects and for the rs16969968 genotype groups (A carrier *vs* GG) are shown in Table 2. rs16969968 was not associated with any baseline smoking-related characteristic.

### CHRNA5 rs16969968\*A Moderates the Aversive Effects of Acute Nicotine

Nicotine increased the ratings of stimulatory effects (main effect for dose:  $F_{(2,3025)} = 105.8$ ,  $P < 0.01$ ) and pleasurable effects (main effect for dose:  $F_{(2,3025)} = 124.7$ ,  $P < 0.01$ ), but not aversive effects (main effect for dose:  $F_{(2,3025)} = 1.85$ ,  $P = 0.16$ ). Relative to rs16969968\*G homozygotes, the aversive effects of IV-administered nicotine were blunted for subjects with one copy of rs16969968\*A (Figure 1; dose-by-group interaction:  $F_{(2,3025)} = 20.49$ ,  $P < 5 \times 10^{-8}$ ). rs16969968 genotype differences in the aversive response to nicotine were evident when EA and AA subjects were analyzed as separate groups (EA dose-by-group interaction:  $F_{(2,1507)} = 17.75$ ,  $P < 5 \times 10^{-8}$ ; AA dose-by-group interaction:  $F_{(2,1506)} = 3.7$ ,  $P < 5 \times 10^{-2}$ ; Supplementary Figure S1) and when the analysis model included three genotype groups (coded additively, rather than A carriers *vs* GG) (dose-by-group interaction:  $F_{(2,3024)} = 21.7$ ,  $P < 5 \times 10^{-8}$ ). The effect remained robust in *post-hoc* analyses that controlled for smoking intensity (cigarettes smoked per day), as well as baseline nicotine metabolite ratio and cotinine and 3-hydroxycotinine levels. The essence of this effect is captured in the bar graph of Figure 1a, which shows the change in the ratings of aversive effects after IV nicotine relative to IV saline for each genotype group. The aversive ratings increased in response to nicotine for the GG group, whereas aversive ratings decrease for rs16969968\*A carriers. In contrast to the pronounced differences in ratings of aversive effects in response to nicotine, there were no compelling differences in ratings of pleasurable effects (dose-by-group interaction:  $F_{(2,3025)} = 2.1$ ,  $P = 0.12$ ) or ratings of stimulatory effects (dose-by-group interaction:  $F_{(2,3025)} = 0.24$ ,  $P = 0.79$ ) for rs16969968\*A carriers relative to rs16969968\*G homozygotes.



**Figure 1** *CHRNA5* rs16969968 moderates the aversive effects of nicotine. The subjective aversive (a), pleasurable (b) and stimulatory (c) ratings of drug effects in response to doses of saline and nicotine for rs16969968\*G homozygotes ( $n = 133$ ) and rs16969968\*A carriers ( $n = 59$ ). The adjacent bar graphs show the change in response to nicotine relative to saline for each drug effect and each genotype group (GG,  $n = 133$ ; AG,  $n = 53$ ; AA,  $n = 6$ ). Mean values are presented ( $\pm$  SEM). 0.5 Nic = 0.5 mg nicotine per 70 kg body weight and 1.0 Nic = 1.0 mg nicotine per 70 kg body weight. \*\*\* $P < 5 \times 10^{-8}$  for the interaction of genotype with dose.

### Cognitive Test Performance

Scores on the CPT, MPT, and Stroop Test were better at the end of the session relative to baseline for each genotype group (main effect for time point for CPT:  $F_{(1,176)} = 74.8$ ,  $P < 0.01$ ; MPT:  $F_{(1,176)} = 54.1$ ,  $P < 0.01$ ; and Stroop Test:

$F_{(1,176)} = 82.6$ ,  $P < 0.01$ ). Subjects with the rs16969968\*A allele showed greater improvement on the Stroop Test relative to rs16969968\*G homozygotes (Figure 2; time point-by-group interaction:  $F_{(1,176)} = 8.11$ ,  $P < 0.01$ ). This effect was consistent when the analysis model included the AA and AG genotype groups (coded additively) rather than A carrier,

and when including smoking intensity (cigarettes smoked per day), nicotine metabolite ratio, and cotinine and 3-hydroxycotinine levels as covariates (time point-by-group interaction:  $F_{(1,167)}=10.1$ ,  $P<0.005$ ). At the end of the session, the average Stroop Test score for the AA genotype group improved 45%, whereas the AG genotype group score improved 31% and the GG genotype group score improved

20%. The score improvement for each cognitive test and each genotype group is shown in Supplementary Figure S2. Test score improvement did not differ by rs16969968 genotype for the CPT (time point-by-group interaction:  $F_{(1,176)}=1.5 \times 10^{-5}$ ,  $P=0.90$ ) or the MPT (time point-by-group interaction:  $F_{(1,176)}=2.26$ ,  $P=0.14$ ). There were no main effects of genotype for CPT, MPT, or the Stroop Test (main effect of genotype:  $P>0.05$ ), including no significant differences at the overnight-abstinence baseline.

**Table 1** Subject rs16969968 Genotype Counts

	rs16969968 genotype count (frequency)			Minor allele frequency	HWE test <sup>a</sup> P-value
	AA	AG	GG		
African American	1 (0.01)	10 (0.10)	85 (0.89)	0.063	0.31
European American	5 (0.05)	43 (0.45)	48 (0.50)	0.28	0.31

<sup>a</sup>Exact test for Hardy–Weinberg equilibrium.

### Nicotine Withdrawal and the Urge to Smoke

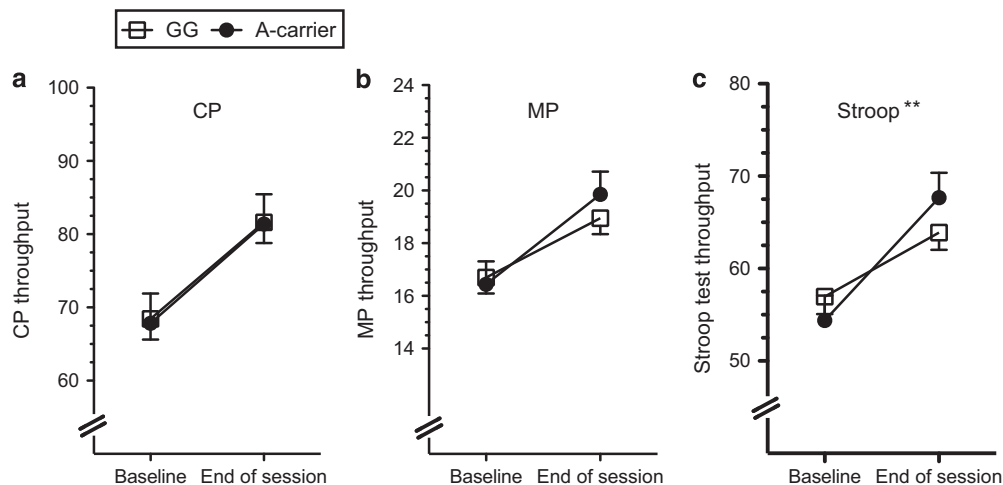
Nicotine withdrawal symptoms (MNWS total score), the urge to smoke for reward (BQSU factor 1), and the urge to smoke to reduce negative symptoms (BQSU factor 2) were lower at the end of the experimental session relative to overnight-abstinence baseline (main effect of time for MNWS:  $F_{(1,188)}=64.85$ ,  $P<0.01$ ; BQSU factor 1:  $F_{(1,184)}=116.6$ ,  $P<0.01$ ; BQSU factor 2:  $F_{(1,184)}=66.6$ ,  $P<0.01$ ). Nicotine withdrawal and the urge to smoke tended to reduce more for rs16969968\*A carriers compared with GG

**Table 2** Smoking-Related Characteristics of the Sample (Mean (SEM))

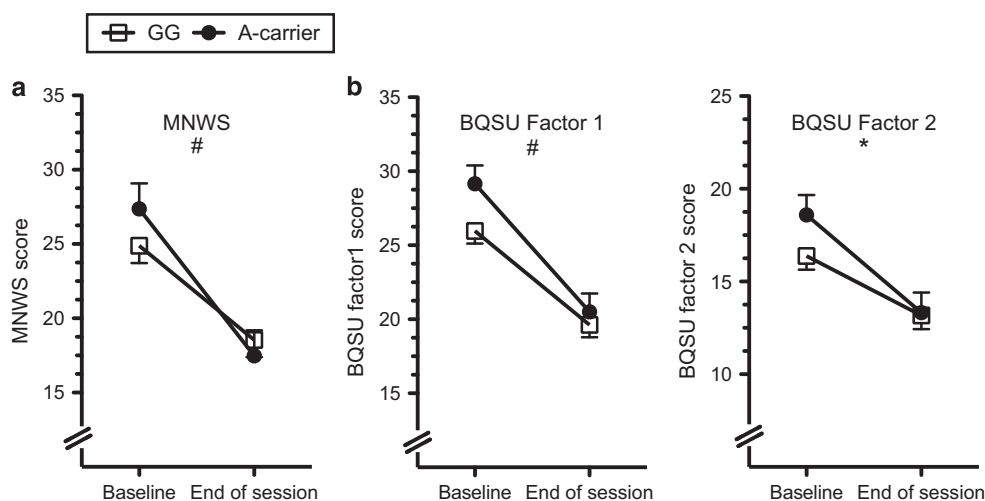
	Cigarettes per day	FTND	Estimated pack years	Longest abstinence (months)	Age of onset	No. of quit attempts	Nicotine (ng/ml)	Cotinine (ng/ml)	NMR
African American	18.55 (1.31)	5.74 (0.20)	17.46 (1.30)	9.24 (2.33)	16.74 (0.41)	3.30 (0.42)	3.43 (0.35)	237.50 (14.50)	0.30 (0.02)
European American	19.13 (1.04)	5.43 (0.21)	19.56 (1.20)	12.42 (2.47)	16.28 (0.49)	5.96 (1.32)	2.29 (0.29)	162.10 (10.69)	0.44 (0.02)
<i>rs16969968 genotype<sup>a</sup></i>									
A carrier ( $n=59$ )	17.98 (1.68)	5.81 (0.29)	17.61 (1.51)	10.80 (3.35)	16.40 (0.62)	6.78 (1.36)	3.23 (0.45)	211.46 (17.84)	0.40 (0.03)
GG ( $n=133$ )	19.08 (1.15)	5.51 (0.20)	19.56 (1.03)	8.62 (2.30)	16.23 (0.43)	3.60 (0.93)	2.55 (0.31)	197.75 (12.22)	0.38 (0.02)

Abbreviations: FTND, Fagerström Test for Nicotine Dependence; NMR, nicotine metabolite ratio.

<sup>a</sup>Mean values did not differ by genotype ( $P>0.05$ ) for all categories adjusted for race, sex, and age with the adjusted means shown.



**Figure 2** *CHRNA5* rs16969968\*A carriers showed greater improvement in the Stroop Task. The throughput scores for (a) The Running Memory Continuous Performance Task (CP), (b) Mathematical Processing Task (MP), and (c) Stroop Task for rs16969968\*G homozygotes and A carriers. Higher throughput scores indicate better performance, with possible scores ranging from 0 to 100 for all three tasks. The mean value adjusted for age, sex, and race is presented ( $\pm$  SEM), and the interactive effect of genotype with time point (baseline vs end of session) is shown. Main effects of genotype were not significant ( $P>0.3$ ) for panels a, b and c. \*\* $P<0.005$  for the interaction of genotype with time point.



**Figure 3** The association of rs16969968 to nicotine withdrawal and the urge to smoke. (a) Minnesota Nicotine Withdrawal Scale (MNWS) scores and (b) Brief Questionnaire of Smoking Urges (BQSU) factor 1 (left) and factor 2 (right) scores, for rs16969968\*G homozygotes and A carriers at baseline and at the end of the session. The mean value adjusted for age, sex, and race is presented ( $\pm$  SEM), and the interactive effect of genotype with time point (baseline vs end of session) is shown. The main effect of genotype was not significant for panels a or b. \* $P < 0.05$  and # $P < 0.1$  for the interaction of genotype with time point.

homozygotes (Figure 3). These trend effects were consistent across measures (time point-by-group interactions for MNWS:  $F_{(1,188)} = 3.13$ ,  $P = 0.079$ ; BQSU factor 1:  $F_{(1,184)} = 2.78$ ,  $P = 0.097$ ; QSU factor 2: ( $F_{(1,184)} = 3.95$ ,  $P = 0.048$ ). There were no main effects of rs16969968 on the MNWS or BQSU (main effect of genotype:  $P > 0.05$ ), and there were no main or interactive associations of rs16969968 with the self-rated positive or negative affect, as measured by the PANAS ( $P > 0.05$ ).

### The Acute Cardiac Response to Nicotine

Nicotine increased HR and diastolic and systolic blood pressure (main effect of dose for HR:  $F_{(2,4096)} = 994$ ,  $P < 0.01$ ; for diastolic blood pressure:  $F_{(2,4115)} = 164.3$ ,  $P < 0.01$ ; for systolic blood pressure:  $F_{(2,4144)} = 224.9$ ,  $P < 0.01$ ). The HR and blood pressure responses to nicotine did not differ for rs16969968 \*A carriers compared with GG homozygotes (main effect of genotype:  $P > 0.05$ ; dose-by-genotype interaction:  $P > 0.05$ ; Supplementary Figure S3).

## DISCUSSION

Variants in the *CHRNA5-A3-B4* nicotinic receptor gene cluster are associated with ND, heavy smoking, and several consequent smoking-related health problems (Amos *et al*, 2008; Berrettini *et al*, 2008; Bierut *et al*, 2007; Bierut *et al*, 2008; Chen *et al*, 2012; Chen *et al*, 2009; Hansen *et al*, 2010; Hung *et al*, 2008; Li *et al*, 2010; Liu *et al*, 2010; Saccone *et al*, 2010; Saccone *et al*, 2007; Sarginson *et al*, 2011; Spitz *et al*, 2008; Thorgeirsson *et al*, 2008; Tobacco and Genetics Consortium, 2010; Wang *et al*, 2009). rs16969968 encodes an amino-acid substitution in *CHRNA5* that might mediate some of these risk effects, and several lines of evidence support this hypothesis. A proposed risk mechanism, based largely on animal models, suggests that the *CHRNA5* risk allele increases the heaviness of smoking by attenuating the aversive response to nicotine (Bailey *et al*, 2010; Bierut *et al*,

2008; Fowler *et al*, 2011; Fowler *et al*, 2013; Frahm *et al*, 2011; Jackson *et al*, 2010; Kuryatov *et al*, 2011; Saccone *et al*, 2010; Saccone *et al*, 2007; Tobacco and Genetics Consortium, 2010). However, this mechanism has not been directly examined in human studies. Here we sought to characterize how rs16969968 might differentially affect the acute response to nicotine in human subjects in the laboratory. We show that the allele associated with heavy smoking, rs16969968\*A reduces the subjective aversive effects of nicotine delivered by IV injection. Although this was not the case in rs16969968\*A carriers, nicotine evoked a pronounced aversive response in subjects who were homozygous for the smoking-protective allele, rs16969968\*G. We extended these findings by showing that rs16969968\*A was also associated with greater improvement in cognitive test performance and consistently lower ratings of withdrawal and urge to smoke following the experimental session. These findings establish an important link between genetic association studies of heavy smoking and smoking-related disorder and the hypothesized risk mechanisms based on functional studies of rs16969968 and behavioral studies of  $\alpha 5$  in rodents. Thus our observations support differential aversion to nicotine as a likely mechanism to explain the association of *CHRNA5* rs16969968 with heavy smoking and disorders related to heavy smoking (Amos *et al*, 2008; Berrettini *et al*, 2008; Bierut *et al*, 2007; Bierut *et al*, 2008; Chen *et al*, 2012; Chen *et al*, 2009; Hansen *et al*, 2010; Hung *et al*, 2008; Li *et al*, 2010; Lips *et al*, 2010; Liu *et al*, 2010; Saccone *et al*, 2010; Saccone *et al*, 2007; Sarginson *et al*, 2011; Spitz *et al*, 2008; Thorgeirsson *et al*, 2008; Tobacco and Genetics Consortium, 2010; Wang *et al*, 2009).

The difference in ratings of aversive effects in response to nicotine for carriers of rs16969968\*A relative to the GG homozygotes is striking, and the specificity of this effect relative to other outcomes is noteworthy. We observed no compelling difference based on rs16969968 for subject ratings of pleasurable effects or stimulatory effects and no differences in cardiovascular reactivity. These observations have several interesting parallels to studies of  $\alpha 5$  function in

rodents, most notably the essential role of  $\alpha 5$  in mediating the aversive effects of nicotine (Bailey *et al*, 2010; Fowler *et al*, 2011; Frahm *et al*, 2011; Jackson *et al*, 2010). The attenuated aversive response to nicotine might reduce the reward-inhibiting and increase the reward-enhancing potential of the drug (Fowler *et al*, 2011; Fowler *et al*, 2013). The lower ratings of aversive effects for A carriers in the nicotine condition relative to the saline condition may relate, partly, to an alleviation of nicotine withdrawal, as shown by the change in BQSU and MNWS scores following nicotine infusion. An experiment conducted with non-abstinent smokers might help to uncouple these effects.

On a measure of selective attention and cognitive control (Stroop task), rs16969968\*A carriers improved more from nicotine-abstinence baseline to end-of-session testing, after they had received nicotine, than GG homozygotes. We found no significant effect of rs16969968 on cognitive function at baseline while participants were in the nicotine-deprivation state (ie, following overnight-nicotine abstinence). Consistent with our baseline findings, a previous study showed no effect of rs16969968 on electrophysiological markers of cognitive control-related processes in nicotine-deprived regular smokers (Evans *et al*, 2014). *CHRNA5* may function in several ways to affect risk for heavy smoking, including by modulating, cognitive function, and specifically the cognitive-enhancing effects of nicotine. The cognitive-enhancing effects of nicotine are an important component of the positive reinforcing effects that may contribute to the development and maintenance of smoking (Newhouse *et al*, 2004). Abstinence-induced differences in cognitive function might also contribute to the maintenance of smoking (Lerman *et al*, 2014). Hong *et al* (2010) showed that rs16969968\*A was associated with weaker connectivity of a dorsal anterior cingulate–ventral striatal circuit and that the strength of this circuit was inversely correlated with ND severity. Consistent with additional roles for *CHRNA5* beyond regulation of aversion to nicotine, Morel *et al* (2014) showed that  $\alpha 5$  expressed in dopaminergic cells of the ventral tegmental area regulates sensitivity to nicotine. Combined, these studies indicate that *CHRNA5* has some pleiotropic effects, beyond regulating aversion to nicotine, that warrant further investigation in relation to risk for heavy smoking.

The well-controlled IV nicotine paradigm was a strength of this study. With this paradigm, we could deliver precise dosing of nicotine to subjects who were biochemically confirmed to be abstinent and evaluate several outcomes that were potentially relevant to the functional effects of rs16969968. The size of the sample, adequate for both EAs and AAs, was also a strength, as we had sufficient power to demonstrate rs16969968 differences for subjective ratings of aversive effects in EA and AA subjects analyzed as separate groups.

Statistical power may have been insufficient to detect effects on some outcomes in the combined sample and in the EA and AA samples analyzed separately. The frequency of rs16969968\*A is considerably higher in EA than in AA populations, and the statistical power to detect effects in the EA subset was far greater compared with the AA subset. There are racial differences, for example, differences in nicotine metabolism (Perez-Stable *et al*, 1998) that influence smoking behavior (Schoedel *et al*, 2004; Tyndale *et al*, 1999; Tyndale and Sellers, 2002). As such, some effects of rs16969968 may differ for EA compared with AA smokers

on account of differences in nicotine metabolism. Our findings, negative and positive, should be viewed in light of these potential differences. Moreover, the Stroop Task and withdrawal effects (MNWS and BQSU) were modest relative to the effects on aversion, and these findings should be viewed as tentative.

Based on the work from several groups (Baker *et al*, 2009; Freathy *et al*, 2009; King *et al*, 2012; Munafo *et al*, 2011; Sarginson *et al*, 2011), including a recent meta-analysis of eight randomized clinical trials of smoking-cessation therapies (Bergen *et al*, 2013), *CHRNA5-A3-B4* variants could be markers for smoking cessation and the response to cessation treatment. The meta-analysis of Bergen *et al* (2013) examined four nAChR SNPs, including rs1051730, which is in almost complete linkage disequilibrium with rs16969968 in European-ancestry populations. Bergen *et al* (2013) found that the minor allele of rs1051730 (in phase with rs16969968\*A) was associated with high rates of abstinence with nicotine replacement therapy (NRT) and lower rates of abstinence with placebo treatment. The mechanisms for this differential treatment response have not been elucidated. Bergen *et al* (2013) suggested that the cognitive enhancing effects of nicotine could potentially mediate better NRT treatment outcomes for individuals with the *CHRNA5* risk allele. Our findings also suggest that the attenuated aversive response to nicotine may help carriers of the *CHRNA5* risk allele (rs16969968\*A) better tolerate NRT. These possibilities need to be further examined in future studies.

In conclusion, we show that rs16969968 has strong, statistically robust, and specific effects on the subjective response to nicotine. These effects may be significant from a translational perspective, and consideration of these effects may help to understand and optimize treatment outcomes, especially treatments based on *CHRNA5-A3-B4* variants. Improving the response to smoking-cessation treatments could have significant health benefit, as quitting, especially at a young age, greatly reduces the mortality associated with smoking (Jha *et al*, 2013).

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