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Association of the histidine-triad nucleotide-binding protein-1 (*HINT1*) gene variants with nicotine dependence

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Abstract

The histidine triad nucleotide-binding protein-1 gene (*HINT1*) is implicated in schizophrenia and in the behavioral effects of morphine and amphetamine. Because nicotine dependence (ND) is highly comorbid with schizophrenia and other substance abuse, we examined the association of *HINT1* with ND. Association analyses from two independent samples show that *HINT1* gene variants are associated with ND phenotypes. Furthermore, human postmortem mRNA expression shows that smoking status and genotype influence *HINT1* expression in the brain. In animal studies, western blot analyses show an increase of *HINT1* protein level in the mouse nucleus accumbens (NAc) after chronic nicotine exposure. This increase was reduced after treatment with the nicotinic-receptor antagonist mecamylamine, and 24 and 72 h after cessation of nicotine treatment. These results indicate a genetic association between *HINT1* variants and ND, and indicate that nicotine-induced modulation of *HINT1* level may be involved in mechanisms of excess smoking.

Keywords

histidine triad nucleotide-binding protein-1 (*HINT1*); single-nucleotide polymorphism; ND; FTND; nucleus accumbens; protein expression

Introduction

The intracellular protein, histidine triad nucleotide-binding protein-1 gene (*HINT1*), is a member of the histidine triad protein family, characterized by a conserved histidine triad sequence motif.[1] Although the *HINT1* protein is widely expressed in liver, kidney and brain, including in the mesocortical and mesostriatal regions,[2] little is known about the physiological function of the protein. The *HINT1* gene is located on chromosome 5q31.2, a region implicated in linkage and association studies of schizophrenia.[3–5] Indeed, *HINT1* microarray analysis identified the gene as a candidate in the neuropathology of schizophrenia. [6,7] Expression studies showed that the level of *HINT1* mRNA was

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Conflict of interest
The authors declare no conflict of interest.

significantly decreased in the prefrontal cortex (PFc) of male schizophrenia patients as compared with that in controls.[8] Additional association and expression studies found variants at *HINT1* to be associated with schizophrenia and also support previous findings suggesting that these associations are sex-specific.[9]

Interestingly, *HINT1* also appears to have a role in modulating the effects of drugs of abuse. Studies examining the CNS function of *HINT1* showed that the protein specifically interacts with the C-terminus of the μ-opioid receptor, leading to attenuation of receptor desensitization and inhibition of PKC-mediated μ-opioid receptor phosphorylation.[10] Furthermore, *HINT1*-knockout mice show enhanced basal and morphine-induced antinociception as well as increased morphine tolerance as compared with their wild-type counterparts.[10] *HINT1*-knockout mice are also hypersensitive to the locomotor-activating effects of amphetamine and the dopamine-receptor agonist apomorphine, suggesting that lack of *HINT1* is associated with dysregulation of postsynaptic dopamine transmission.[11]

Nicotine dependence (ND) is highly comorbid with schizophrenia[12] and other substance abuse. Despite studies suggestive of a role for *HINT1* in both schizophrenia and mechanisms of drug dependence, to date there are no available studies examining the role of *HINT1* in ND. Thus, this study determined the role of the *HINT1* gene in ND. We propose that variants at *HINT1* are associated with ND and that nicotine induces changes in *HINT1* protein level in brain regions relevant to mechanisms of ND. Using *HINT1* markers selected for and found to be significantly associated with schizophrenia in a previous study at our laboratory,[9] we performed association studies of the *HINT1* gene using ND phenotypes. In addition, we analyzed *HINT1* gene expression in human postmortem brain samples obtained from smokers and non-smokers, and measured *HINT1* protein levels in the mouse PFc, hippocampus, nucleus accumbens (NAc) and ventral tegmental area (VTA) after acute and chronic nicotine exposure, after pretreatment with the non-selective nicotinic acetylcholine receptor antagonist, mecamylamine, and 24 and 72h after cessation of nicotine treatment.

Materials and methods

Human subjects and measurements

In this study, we used two population samples. One was selected from the Virginia Adult Twin Study of Psychiatric and Substance Use Disorder,[13,14] referred to as VA-Twins hereafter. In this sample, only regular smokers (defined as those who smoked at least one cigarette per day for at least 4 weeks at some point in their life) were included. Tobacco smoking and ND were assessed by the Fagerström Tolerance Questionnaire and/or the Fagerström Test of ND (FTND) during the time of heaviest lifetime nicotine use.[15,16] For each twin pair, only one twin was selected. All subjects in the sample were adults between the ages of 18 and 65 at the time of FTND ascertainment, and all were of self-reported European ancestry. The second sample used in this study included the control subjects from the Genetic Association Information Network (GAIN) organization. We obtained the GAIN data set through NCBI dbGaP (http://www.ncbi.nlm.nih.gov/gap). For the GAIN subjects, we used only those who self-reported as Caucasian. The GAIN controls had a variety of psychiatric and substance abuse evaluations, including the FTND questionnaire.

We used three ND measurements in this study. One was the FTND score, which was a quantitative phenotype. The second was a dichotomized ND phenotype in which subjects with low ND (FTND scores ≤3) were used as controls and those with FTND scores ≥6 were used as cases. The third phenotype we used was a categorized number of cigarettes smoked per day (numCIG). Based on the distribution of the raw data of the number of cigarettes smoked per day in the two samples used in this study, we selected four cut-off thresholds: 1, 2–15, 16–30 and ≥31. The VA-Twins included 2086 subjects with FTND scores, of which 846 had FTND scores ≤3 (controls, mean FTND score=1.6, s.d.=1.06) and 771 had FTND scores ≥6 (cases, mean FTND score=7.3, s.d.=1.12). In the GAIN sample, we used 973 subjects with appropriate smoking data, of which 700 had FTND scores. The number of subjects for the four numCIG categories in the VA-Twins sample was 15, 426, 1015 and 645. In the GAIN study, for the ND phenotype there were 239 cases and 267 controls, and there were 148, 295, 360 and 170 subjects respectively for the four numCIG categories.

Marker selection, genotyping and imputation

HINT1 markers were selected as described by Chen *et al*.[9] and this was done on the basis of significant association with schizophrenia in the Chen *et al*.[9] study. Four single-nucleotide polymorphisms (SNPs) were typed for VA-Twins. Genotyping was conducted using the Taqman Method[17] and genotypes were scored using a semi-automated Excel Template developed in our laboratory.[18] All typed SNPs were checked for Hardy–Weinberg equilibrium using the HAPLOVIEW program.[19] The GAIN controls were typed by an Affymetrix microarray.[20] Of the four SNPs typed in the VA-Twins, two (rs2551038 and rs2526303) were included in the Affymetrix 6.0 chip set. By checking data from the HapMap database (http://hapmap.ncbi.nlm.nih.gov/), we found that rs3864283 was typed in the HapMap project; therefore, we downloaded the HapMap phase-3 data set and imputed the genotypes for rs3864283 for the GAIN controls using the fastPHASE program.[21]

Human postmortem mRNA expression studies

Expression studies were performed using postmortem brain cDNAs from the Stanley Medical Research Institute (http://www.stanleyresearch.org) and mRNA isolation and reverse transcription were performed by Stanley Researchers. The Stanley panel consisted of 104 subjects. Thirty-five individuals were diagnosed with schizophrenia, 34 with bipolar disorder and 35 were unaffected controls. For this study, we used only the unaffected control subjects to analyze the influence of smoking status and genotype on *HINT1* expression. Of the 35 control subjects, 14 had smoking status information (7 smokers and 7 non-smokers).

Q-PCRs were conducted as described by Chen *et al*.[9] In brief, the TaqMan expression probe, Hs00602163_m1, was used to analyze the *HINT1* gene and the human TATA box-binding protein (*TBP*) gene was used as an internal reference. Each sample was amplified in triplicates, with 0.25 ng of cDNAs used in 15 μl of PCR mixture containing the FAM-labeled *HINT1* probe and the VIC-labeled *TBP* probe. PCR was conducted and the expression level of each reaction was determined by the C_T value. The results from three repeat assays were averaged to produce a single mean C_T value for each individual. The relative expression level between *HINT1* and *TBP* for each individual was calculated by the $2_T^{\Delta C}$ method, where $\Delta C_T = C_T^{HINT1} C_T^{TBP}$.[22]

Animals

Male 129SvJ mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The animals were 8–10 weeks of age at the start of the studies and were group-housed in a 21 °C humidity-controlled animal care facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care, with *ad libitum* access to food and water. Four mice per group were used for all experiments. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and in accordance with the National Institutes of Health Guide for Animal Care and Use.

Drugs

(−)-Nicotine hydrogen tartrate salt and mecamylamine hydrochloride were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Drugs were dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously (s.c.) at a volume of 10 ml kg^{-1} body weight. Doses are expressed as the free base of the drug.

Western blot assay for *HINT1*

Mice were treated with acute or chronic nicotine. PFc, NAc, VTA and hippocampal sections were dissected and homogenized in cold extraction buffer containing 50mM Tris, 1% sodium dodecyl sulfate, 1mM phenylmethylsulfonyl fluoride, 1mM EDTA, 5mM EGTA, 1mM Na^{+} orthovanadate, 20 $\mu g ml^{-1}$ leupeptin, 10 $\mu g ml^{-1}$ aprotinin and 1 μM okadaic acid. Protein concentrations were determined using the Bradford assay and 30 μg of protein were incubated with 6X blue gel loading dye (New England Biolabs, Ipswich, MA, USA) and heated for 5 min at 95 °C. The samples were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 15% Tris-HCl gel and subjected to immunoblotting. Non-specific protein was blocked in 5% milk solution in TPBS for 1 h at room temperature. An *HINT1* primary antibody (1:1000; Proteintech Group Inc., Chicago, IL, USA) and an α-tubulin antibody (1:10000; Upstate, Temecula, CA, USA) were incubated overnight at 4 °C. Secondary antibodies (1:5000; LiCor Biosciences Inc., Lincoln, NE, USA) were incubated for 1 h at room temperature. Bound antibody was detected using the LiCor Odyssey Infrared Imaging System (LiCor Biosciences Inc., Lincoln, NE, USA). *HINT1* bands were detected at 14 kDa and α-tubulin bands were detected at 55 kDa.

Acute studies

Mice were treated with a single injection of saline or nicotine (0.5, 1 or 2$mg kg^{-1}$, s.c.) and killed by cervical dislocation 20 min after the injection. Brain sections were dissected and placed immediately in cold extraction buffer.

Chronic nicotine administration protocol

Mice were anesthetized with sodium pentobarbital (45 kg kg^{-1} by intraperitoneal injection) and implanted s.c. with Alzet osmotic mini pumps (model 2004; Durect Corporation, Cupertino, CA, USA) filled with (−)-nicotine or saline solution as described by Jackson *et al.*[23] The concentration of nicotine was adjusted according to animal weight and mini pump flow rate so that mice were infused with 36$mg kg^{-1}$ day^{-1} for 14 days. The dose and duration

of nicotine exposure were chosen on the basis of previous behavioral studies,[23,24] which show that significant tolerance and nicotine withdrawal signs are produced in mice after this treatment regimen.

Chronic studies

Mice were chronically infused with nicotine for 14 days as described in the chronic administration protocol. On the morning of day 15, mice were killed by cervical dislocation. Brain sections were dissected and placed immediately in cold extraction buffer.

Withdrawal studies

Chronic nicotine- and saline-infused mice were injected with a non-selective nicotinic-receptor antagonist, mecamylamine ($2 mg kg^{-1}$, s.c.), on the morning of day 15. Mice were killed by cervical dislocation 30 min after the injection. Brains were dissected and placed in cold extraction buffer. For spontaneous withdrawal studies, mini pumps were removed on day 14. Twenty-four hours later on day 15 or 72 h later on day 18, mice were killed by cervical dislocation and brain sections were removed and prepped for western blot analysis.

Statistical analyses

Statistical analyses for human association studies were conducted using the PLINK program.[25] In these analyses, ND phenotype was treated as case control and analyzed by logistic regression, and FTND scores and numCIG were treated as quantitative traits and analyzed by linear regression. In all association analyses, sex and age at interview were used as covariates. For haplotype analysis, we used the proxy association function of the PLINK program where rs2526303 was used as the target SNP. Correction for multiple comparisons was applied to all significant results; however, the *P*-values reported in the paper are the uncorrected values. For samples from the Stanley Institute, smoking status (current smokers) and rs3864283 genotype were tested for association with *HINT1* expression. Before the final analysis, individual factors, such as sex, brain pH, lifetime substance usage and lifetime alcohol usage, were evaluated for their influences on *HINT1* mRNA expression. The analyses were conducted using the univariate general linear model implemented in the SPSS software (version 17 for Windows). For western blot studies, statistical analyses were performed using StatView. Data were analyzed using one-way analysis of variance with treatment as the between-subject factor. Significant results were further assessed using Neuman–Keuls *post hoc* test. For expression studies, *P*-values less than 0.05 were considered significant.

Results

Association analysis of *HINT1*

The VA-Twins were genotyped using the four markers found to be significantly associated with schizophrenia in the Chen *et al*. study: rs2189663, rs2551038, rs3864283 and rs2526303.[9] The GAIN sample was typed for rs2551038 and rs2526303, and imputed for rs3864283. The association analysis results are shown in Table 1. Single-marker analysis showed that two markers (rs3864283 and rs2526303) were nominally significant for all three phenotypes or showed a trend in the VA-Twins. For the GAIN controls, rs3864283

showed the same direction of association for all three phenotypes; however, due to the small sample size and potential power issues, none of these were significant. When the two samples were combined, rs3864283 and rs2526303 reached experiment-wide significance for numCIG when all phenotypes and markers were corrected for multiple testing (P<0.05/12 or 0.0042). Subjects carrying the major alleles (T and G respectively for the two markers) had higher FTND scores and numCIG as compared with those carrying the minor alleles (C and A alleles). Using the proxy association test function of the PLINK program, we identified the risk haplotype GTG for the markers rs2551038–rs3864283– rs2526303 (Table 2). In these analyses, the results for both FTND and numCIG were consistent and significant.

Analysis of *HINT1* mRNA expression in postmortem brain

Q-PCR was used to determine *HINT1* mRNA expression in 14 postmortem brain samples (7 smokers, 7 non-smokers). Before final analysis, individual factors, such as sex, brain pH, lifetime substance usage and lifetime alcohol usage, were evaluated for their influences on *HINT1* mRNA expression. In these analyses, none of these factors were found to be correlated with *HINT1* mRNA levels in the brain; therefore, they were not included as covariates in the final analysis. The results are shown in Table 3. Smoking status itself was not significantly associated with *HINT1* mRNA expression; however, there was a trend for smokers to have higher expression than non-smokers (relative expression mean ± s.d.: smokers 7.07 ± 1.99; non-smokers 5.71 ± 4.29). Alternatively, the rs3864283 genotype was found to be nominally significant in the association with *HINT1* mRNA expression. When smoking status and rs3864283 were considered together, they were significantly associated with *HINT1* mRNA (P=0.0046).

HINT1 protein expression after acute nicotine exposure

HINT1 mRNA expression studies showed that smoking status and genotype jointly influence *HINT1* expression in the human brain. Smokers also showed a trend toward higher *HINT1* expression in the brain than non-smokers. To examine this effect further, we determined whether *HINT1* protein level is altered in response to nicotine treatment. Mice (n=4 per group) were given a single injection of nicotine (0.5, 1 or 2mg kg^{-1}, s.c.) and the PFc, hippocampus, NAc and VTA were removed after 20min. Western blot analysis showed no significant difference between the saline- and nicotine-treated groups in any brain section tested, indicating that *HINT1* level in these brain areas is unchanged after acute nicotine exposure (Supplementary Figure 1).

HINT1 protein level after chronic nicotine exposure

To examine the effect of chronic nicotine on *HINT1* protein level, mice (n=4 per group) were chronically infused with nicotine or saline through subcutaneously implanted osmotic mini pumps for 14 days. Brain dissections were taken the morning of day 15 for chronic studies, or 30 min after injection with the non-selective nicotinic-receptor antagonist, mecamylamine (2mgkg^{-1}, s.c.). The results are shown in Supplementary Figure 2 (PFc, hippocampus and VTA) and in Figure 1 (NAc). Chronic nicotine significantly increased *HINT1* protein level in the NAc ($F_{(3,44)}$=25.85, P<0.005; Figure 1a). This increase in

expression was not present in the PFc, hippocampus or VTA. The chronic nicotine-induced increase in the *HINT1* level observed in the NAc was significantly reduced after mecamylamine treatment ($F_{(3,44)}$=25.85, P<0.05; Figure 1a).

A time course of *HINT1* protein level after cessation of nicotine treatment was also conducted. Mini pumps were removed and *HINT1* level was measured in the brain areas 24 and 72 h after withdrawal from nicotine. Similar to what was observed in the precipitated assessment, chronic nicotine induced an increase in the *HINT1* level in the NAc ($F_{(5,66)}$=4.853; P<0.05; Figure 1b), and this increase returned to baseline levels 24 h after mini pump removal (Figure 1b). There was no significant difference between the 24- and 72-h time points. No change was observed in any other brain region after withdrawal from nicotine (Supplementary Figure 3), indicating that the observed effects are specific to the NAc.

Discussion

Results from association, expression and molecular studies suggest that *HINT1* is involved in mechanisms of ND. The association studies using the VA-Twins and GAIN controls showed that two markers (rs3864283 and rs2526303) were significantly associated with ND, specifically the FTND scores and numCIG, suggesting that the gene is associated with increased risk of ND. In addition, *HINT1* mRNA expression levels in the brain showed a tendency toward higher expression in smokers than in non-smokers. Although these results did not reach significance, it may be due to the small sample size used in the expression study, as only 14 healthy subjects with smoking information were available. Interestingly, the main effects of genotype suggest that individuals with the specific polymorphism, which was found to be associated with FTND score and numCIGs, have significantly higher *HINT1* expression levels, whereas the genotype (rs3864283) × smoking status interaction suggests that this effect is dependent on the smoking status, that is smokers with the risk allele of rs3864283 have higher *HINT1* expression than non-smokers. These results are in support of our hypothesis that variants at *HINT1* are associated with mechanisms of ND, as a change in mRNA expression in smokers may suggest that the polymorphism has some biological relevance in the development of ND.

In vivo protein expression studies showed that *HINT1* protein level was not altered in any brain region tested after a single injection of nicotine; however, consistent with our human brain expression studies, there was a significant increase in the protein level in the NAc after chronic nicotine exposure, and this increase was reduced after a single injection of the non-selective nAChR antagonist, mecamylamine, suggesting that the chronic nicotine-induced increase in the *HINT1* level is mediated directly through nAChRs. Furthermore, the nicotine-induced changes in the *HINT1* level were only observed in the NAc, indicating a brain-region-specific effect. Indeed, the NAc is a brain region implicated in ND behaviors, including nicotine reward,[26–28] self-administration[29] and withdrawal. [27,30] On the basis of these observations, it can be stated that the nicotine-induced increase in *HINT1* is mediated through specific nAChR subtypes such as α4β2* (where * denotes the possible incorporation of additional subunits) or α7, the major nAChR subtypes in the brain, which have been shown to mediate behaviors associated with ND. It cannot be ruled out that the

changes observed in the *HINT1* level specifically in the NAc after cessation of nicotine treatment have some relevance in ND. Future studies will elucidate the specific nAChR subtypes involved in the nicotine-induced increases in the *HINT1* level, and examination of involvement of *HINT1* in post-receptor pathways relevant to ND.

The direct purpose of this study was to test whether *HINT1* is involved in mechanisms of ND. This study was designed on the basis of previous reports that *HINT1* variants are associated with schizophrenia,[9] *HINT1* mRNA expression is decreased in the postmortem brain tissues of schizophrenia patients,[8] *HINT1*-knockout mice have altered responses to morphine[10] and amphetamine,[11] and evidence that ND is highly comorbid with both schizophrenia and substance abuse/dependence.[12] Results from three independent studies suggest that, indeed, *HINT1* is involved in ND. As discussed above, the markers selected in this study were all associated with schizophrenia in a previous study;[9] however, the relationship between *HINT1*, ND and schizophrenia seems more complicated. In that study, the risk alleles for schizophrenia for female subjects were C and A for rs3864283 and rs2526303 respectively. In this study, these same alleles of the same markers were protective in ND. Similarly, in haplotype analyses, the haplotype G-C-A for SNPs rs2551038–rs3864283–rs25326303 is the risk haplotype for schizophrenia, but this same haplotype is protective in ND (see Table 2). In other words, whereas the same markers showed significant association with both schizophrenia and ND, the effects of association were in the opposite direction. How alleles promoting smoking in the general population protect schizophrenia patients is not clear. Further studies with samples of dual phenotypes are required to clarify these differences.

Overall, the results of this study, taken together with previous studies, suggest that *HINT1* is an important genetic component associated with mechanisms of ND, with potential implications in understanding the comorbidity between ND and schizophrenia, and the genetic mechanisms relevant to drug dependence in general.

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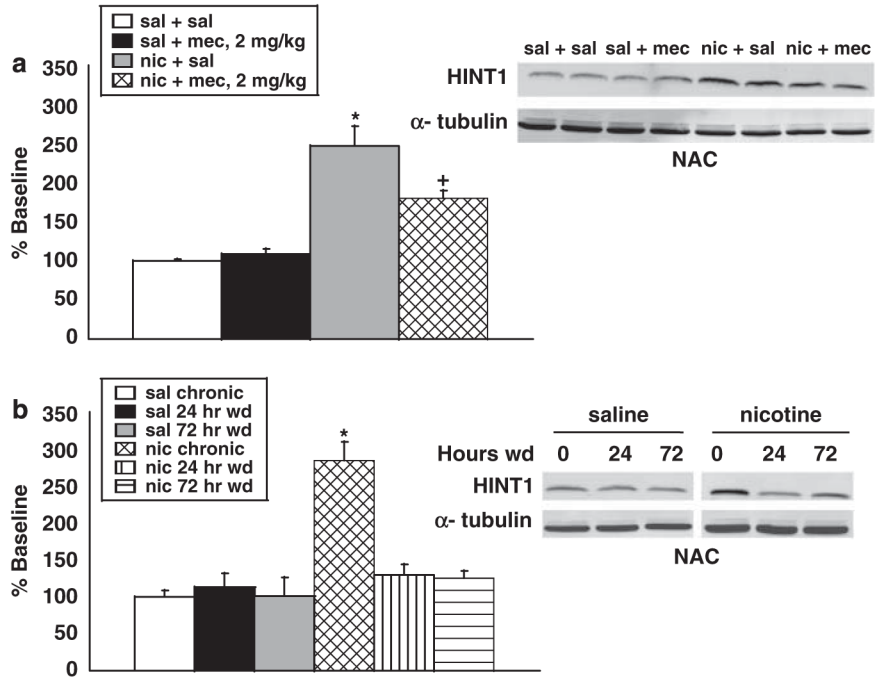

Figure 1.

HINT1 protein levels are increased in the NAc after chronic nicotine exposure and reduced after mecamylamine treatment and nicotine withdrawal. Mice were chronically infused with nicotine ($36 mgkg^{-1}day^{-1}$) for 14 days. Brain sections were dissected on the morning of day 15, 30 min after saline or mecamylamine ($2 mgkg^{-1}$, s.c.) injection. Chronic nicotine induced a significant increase in the HINT1 level in the NAc (**a**, **b**). (**a**) The chronic nicotine-induced increase in the HINT1 level in the NAc was reduced after mecamylamine treatment. (**b**) The chronic nicotine-induced increase in the HINT1 level in the NAc returned to baseline levels 24 and 72 h after nicotine withdrawal. The *y*-axis of the graph represents the percent change from baseline (saline). Each point represents the mean ± s.e.m. of four mice per group. Results were pooled from three separate experiments for each brain section. * denotes $P<0.05$ versus saline baseline and + denotes $P<0.05$ versus the chronic nicotine and saline groups. α-tbn, α-tubulin; HINT1, histidine-triad nucleotide-binding protein-1; NAc, nucleus accumbens; nic, nicotine; Sal, saline; s.c., subcutaneous.

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Table 1

Single-marker association with ND and FTND

SNP	Allele	VA-Twins (*n*=2113)			GAIN controls (*n*=973)			Combined (*n*=3086)		
		OR/Beta	*STAT*	*P*	OR/Beta	STAT	*P*	OR/Beta	STAT	*P*
ND										
rs2189663	A	0.92	−1.06	0.2909	—	—	—	0.92	−1.06	0.2909
rs2551038	C	0.86	−1.31	0.1893	1.31	1.39	0.1660	0.95	−0.52	0.6050
rs3864283	C	0.86	−1.78	0.0748	0.96	−0.25	0.8019	0.89	−1.65	0.0983
rs2526303	A	0.85	−2.21	**0.0271**	1.11	0.76	0.4463	0.91	−1.52	0.1293
FTND										
rs2189663	A	−0.13	−1.42	0.1568	—	—	—	−0.13	−1.38	0.1664
rs2551038	C	−0.17	−1.33	0.1847	0.27	1.32	0.1884	−0.06	−0.55	0.5847
rs3864283	C	−0.19	−2.01	**0.0446**	−0.06	−0.36	0.7221	−0.17	−1.99	**0.0462**
rs2526303	A	−0.18	−2.12	**0.0342**	0.06	0.39	0.6956	−0.13	−1.72	0.0850
numCIG										
rs2189663	A	−0.01	−0.54	0.5918	—	—	—	−0.01	−0.54	0.5918
rs2551038	C	−0.03	−0.93	0.3541	0.03	0.42	0.6774	−0.03	−0.79	0.4314
rs3864283	C	−0.05	−1.86	0.0637	−0.06	−1.13	0.2584	−0.07	−2.94	**0.0033**
rs2526303	A	−0.05	−2.18	**0.0295**	−0.03	−0.74	0.4572	−0.07	−3.07	**0.0022**

Abbreviations: FTND, Fagerström Test of ND; ND, nicotine dependence; numCIG, number of cigarettes smoked per day; OR, odds ratio; SNP, single-nucleotide polymorphism; VA-Twins, Virginia Adult Twin Study of Psychiatric and Substance Use Disorder.

Single-marker analysis of the VA-Twins and GAIN controls. ND was the dichotomized phenotype. FTND and numCIG were used as the quantitative phenotypes.

P values<0.05 are underlined in bold.

Table 2

Haplotype analysis for ND and FTND

Haplotype	Freq	OR/Beta	Chisq/STAT	*P*
ND				
GCA	0.227	0.89	2.48	0.1150
CTA	0.116	0.94	0.38	0.5370
GTA	0.026	1.21	0.99	0.3210
GCG	0.024	0.91	0.19	0.6600
GTG	0.604	1.10	2.09	0.1480
FTND				
GCA	0.227	−1.67	8.86	**0.0029**
CTA	0.116	−0.33	0.20	0.6530
GTA	0.026	0.56	0.15	0.7020
GCG	0.024	−0.64	0.17	0.6840
GTG	0.604	1.29	7.31	**0.0069**
numCIG				
GCA	0.227	−0.08	9.66	**0.0019**
CTA	0.116	−0.03	0.72	0.3960
GTA	0.026	0.04	0.42	0.5190
GCG	0.024	−0.01	0.03	0.8590
GTG	0.604	0.06	7.75	**0.0054**

Abbreviations: Chisq, χ^2-test; Freq, frequency; FTND, Fagerström Test of ND; ND, nicotine dependence; numCIG, number of cigarettes smoked per day; OR, odds ratio.

Three-marker (rs2551038–rs3864283–rs2526303) haplotype association analysis of the VA-Twins and GAIN controls.

P values<0.05 are underlined in bold.

Table 3

Smoking status and rs3864283 influence *HINT1* mRNA expression

Source	df	F	*P*
Corrected model	3	6.838	0.0087
Intercept	1	90.821	0.0000
Smoking	1	3.115	0.1081
rs3864283	1	5.964	**0.0347**
Smoking * rs3864283	1	13.157	**0.0046**

Abbreviation: HINT1, histidine-triad nucleotide-binding protein-1.

Q-PCR analysis of *HINT1* mRNA expression in postmortem brain samples (n=14; 7 smokers, 7 non-smokers).

P-values<0.05 are underlined in bold.