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The Neuropeptide Galanin and Variants in the *GalR1* Gene are Associated with Nicotine Dependence

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The neuropeptide galanin and its receptors are expressed in brain regions implicated in drug dependence. Indeed, several lines of evidence support a role for galanin in modulating the effects of drugs of abuse, including morphine, cocaine, amphetamine, and alcohol. Despite these findings, the role of galanin and its receptors in the effects of nicotine is largely underexplored. Here, using mouse models of nicotine reward and withdrawal, we show that there is a significant correlation between mecamylamine-precipitated nicotine withdrawal somatic signs and basal galanin or galanin receptor 1 (GALR1) expression in mesolimbocortical dopamine regions across the BXD battery of recombinant inbred mouse lines. The non-peptide galanin receptor agonist, galnon, also blocks nicotine rewarding effects and reverses mecamylamine-precipitated nicotine withdrawal signs in ICR mice. Additionally, we conducted a meta-analysis using smoking information from six European-American and African-American data sets. In support of our animal data, results from the association study show that variants in the *GALR1* gene are associated with a protective effect in nicotine dependence (ND). Taken together, our data suggest that galanin has a protective role against progression to ND, and these effects may be mediated through GALR1.

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INTRODUCTION

The neuropeptide galanin is widely expressed in the central nervous system and is involved in modulation of food and alcohol intake, cognition, depression, and anxiety disorders (Holmes *et al*, 2003; Leibowitz, 2005; Rustay *et al*, 2005). The CNS effects of galanin are mediated through the galanin receptors, GALR1, GALR2, and GALR3. All three receptors are expressed in brain areas implicated in drug dependence, including the ventral midbrain (VMB, which includes the ventral tegmental area), substantia nigra, nucleus accumbens (NAc), and locus coeruleus (LC) (Mennicken *et al*, 2002). Indeed, emerging evidence implicates a role for galanin in altered responses to drugs of abuse.

Galanin modulates the mesolimbic dopamine system by inhibiting dopaminergic transmission (Tsuda *et al*, 1998; Ericson and Ahlenius, 1999), an effect necessary for the rewarding properties of drugs of abuse (Koob, 1992). Galnon, a non-peptide galanin receptor agonist, attenuates morphine preference and withdrawal in the mouse (Zachariou *et al*, 1999, 2003). In addition, mice overexpressing the galanin peptide in noradrenergic neurons show decreased morphine withdrawal signs, while galanin knockout ($Gal^{-/-}$) mice show increased withdrawal signs (Zachariou *et al*, 2003). $Gal^{-/-}$ mice also show greater preference for cocaine, an effect reversed by galnon administration (Narasimhaiah *et al*, 2009). Furthermore, transgenic mice that overexpress galanin in the brain are less sensitive to amphetamine-induced increases in locomotor activity (Kuteeva *et al*, 2005). These data suggest that galanin has a protective role against progression to drug dependence. Alternatively, administration of the galanin antagonist, M40, into the paraventricular nucleus reduces alcohol intake in rats (Rada *et al*, 2004), a behavior also reduced in Gal $^{-/-}$ mice (Karatayev *et al*, 2010). In support of this animal data, human studies identified variants in the galanin and *GALR3* genes associated with risk for alcoholism (Belfer *et al*, 2006, 2007). It has been suggested that this discrepancy between the effects of galanin in alcohol *vs* other drugs of abuse could be attributed to the caloric content of alcohol. Consequently, peptides regulating feeding behavior may have more complex effects on alcohol intake (Picciotto, 2008). Despite the differences, a role for galanin in modulating drug dependence behaviors is evident.

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Currently, the role of galanin and its receptors in the effects of nicotine is largely underexplored. In this study, we investigated this role by using mouse and human genetic approaches. We first characterized nicotine withdrawal in two inbred strains of mice, the C57BL/6J (B6) and DBA/2J (D2) strains, which have markedly different responses to various drugs of abuse, including nicotine (Jackson *et al*, 2009), and 25 of the BXD (B6 X D2) recombinant inbred strains to enable genetic correlation analyses for identification of genes showing strong correlations to variation in nicotine behavioral phenotypes. We then tested the effects of galnon, a non-peptide GALR1 and GALR2 agonist, in mouse models of nicotine reward and withdrawal. We finally complemented our mouse studies with a genetic association study and meta-analysis to examine possible associations between *GalR* genes and nicotine dependence (ND).

MATERIALS AND METHODS

Animals

Naïve male C57BL/6J mice, DBA/2J mice, and their recombinant strains (BXD) were obtained from Jackson Laboratories (Bar Harbor, ME). Naïve male ICR mice were purchased from Harlan Laboratories (Indianapolis, IN). Animals were 8–10 weeks of age at the start of the experiments and were group housed in a 21 °C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility with *ad libitum* access to food and water. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs

(−)-Nicotine hydrogen tartrate salt, mecamylamine hydrochloride, and galnon trifluoroacetate salt were purchased from Sigma-Aldrich (St Louis, MO). Nicotine was dissolved in physiological saline (0.9% sodium chloride), and galnon was dissolved in a 5% dimethyl sulfoxide (DMSO) solution. Nicotine was injected subcutaneously (s.c.) and intraperitoneal (i.p.) injection was used to administer galnon at a volume of 10 ml/kg body weight unless noted otherwise. All doses are expressed as the free base of the drug.

Nicotine Conditioned Place Preference (CPP)

An unbiased CPP paradigm was utilized in this study as a model of nicotine reward as described in Kota *et al* (2007). Briefly, place conditioning chambers consisted of two distinct compartments separated by a smaller intermediate compartment with openings that allowed access to either side of the chamber. On day 1, animals were confined to the intermediate compartment for a 5-min habituation period, and then allowed to move freely between compartments for 15 min. Time spent in each compartment was recorded. These data were used to separate the animals into groups of approximately equal bias. Days 2–4 were the conditioning days during which mice were pretreated with vehicle (5% DMSO, i.p.) or galnon (i.p.) 15 min before saline and nicotine s.c. injections. Galnon-treated groups received doses of 0.01, 0.02, or 0.2 mg/kg before nicotine. The saline group received saline in both compartments and drug groups received nicotine (0.5 mg/kg, s.c.) in one compartment and saline in the opposite compartment. Drug-paired compartments were randomized among all groups. Day 5, test day, mice did not receive any drug. Activity counts and time spent on each side were recorded via photosensors using Med Associates interface and software. Data are expressed as time spent on the drug-paired side minus time spent on the saline-paired side. A positive number indicates a preference for the drug-paired side, whereas a negative number indicates an aversion to the drug-paired side. A number at or near zero indicates no preference for either side.

Nicotine Chronic Administration and Withdrawal

Mice were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and implanted with Alzet osmotic mini pumps ((model 1007D) Durect Corporation, Cupertino, CA) filled with (−)-nicotine (24 mg/kg per day) or saline solution for 7 days. The concentration of nicotine was adjusted according to animal weight and mini pump flow rate. For withdrawal studies, mice were injected with vehicle (5% DMSO, i.p.) or galnon (0.5 mg/kg, i.p.) 15 min before initiation of testing on day 8. The non-selective nicotinic acetylcholine receptor (nAChR) antagonist, mecamylamine (2 mg/kg, s.c.), was administered 5 min after vehicle or galnon, and 10 min before initiation of testing to precipitate withdrawal signs. The withdrawal testing sequence was as described in Jackson *et al* (2008). Affective (anxiety-like behavior) and physical (somatic signs, hyperalgesia) nicotine withdrawal signs were evaluated in this paradigm. Mice were first evaluated for 5 min in the plus maze test for anxiety-related behavior, followed by a 20-min observation of somatic signs measured as paw and body tremors, head shakes, backing, jumps, curls, and ptosis. Hyperalgesia was evaluated using the hot plate (52 °C) immediately following the somatic sign observation period.

Galanin-Related Gene Expression Correlation with Nicotine Withdrawal Somatic Signs in BXD Inbred Panel

Ongoing mouse genetic studies have studied phenotyping of nicotine behavioral data across the BXD recombinant inbred mouse panel. These studies enable genetic correlation analyses for nicotine behaviors, possibly identifying quantitative trait loci. In addition, by correlating such extensive behavioral data with existing whole genome expression studies, we can identify genes showing strong correlations to variation in nicotine behavioral phenotypes. We used this approach to identify candidate genes possibly modulating behavioral responses to nicotine. BXD mice were infused through osmotic minipumps with nicotine (24 mg/kg per day) for 7 days, and withdrawal signs were analyzed on day 8, 10 min after a single injection of mecamylamine (2 mg/kg, s.c.) or saline as described in the previous section (nicotine chronic administration and withdrawal). BXD behavioral data for somatic signs, after chronic nicotine followed by saline or mecamylamine

injection, were correlated with existing microarray expression data for galanin and GalRs 1–3 from multiple data sets within GeneNetwork (http://www.genenetwork.org). These data sets included: NAc (VCU BXD NA Sal M430 2.0 RMA); VMB (VCU BXD VTA Sal M430 2.0 RMA); dorsal striatum, DST (HQF BXD Striatum ILM6.1 (Dec10v2) RankInv); hippocampus, HPC (Hippocampus Consortium M430v2 (Jun06) RMA); amygdala, AMG (INIA Amygdala Affy MoGene 1.0 ST (Nov10) RMA); and whole brain (UCHSC BXD Whole Brain M430 2.0 (Nov06) RMA). All comparisons used the genetic Pearson product moment correlations within GeneNetwork. Similar correlations were also done for somatic sign difference scores (Mec-induced – saline-induced) and the vehicle control somatic signs scores (Sal-induced).

Human Subjects and Measurements

All data sets used in this study were obtained via the NCBI dbGAP website (http://www.ncbi.nlm.nih.gov/gap) under a protocol approved by the Virginia Commonwealth University Institutional Review Board and the National Institutes of Health. The population samples used were the European-American and African-American control subjects from the Molecular Genetics of Schizophrenia (MGS) genome-wide association study and subjects of European-American ancestry from the Collaborative Genetic Study of Nicotine Dependence (COGEND), the Collaborative Study on the Genetics of Alcoholism (COGA), the Family Study of Cocaine Dependence (FSCD), and the Genome-Wide Scan of Lung Cancer and Smoking Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO). Seven markers in the *GALR1* gene or within 4 kb of the gene were included in this study. Marker positions, nucleotide variation, and allele frequency are shown in Table 1. In the COGEND, COGA, and FSCD studies, the markers rs9959924 and rs11877007 were imputed using the fastPHASE program (Scheet and Stephens, 2006) and the MGS European-American sample as a reference panel, as this data set contains a larger number of subjects than the Hapmap panel, an advantageous factor in increasing the accuracy and consistency of the imputation. In the PLCO study, the markers rs9959924, rs11877007, and rs2717164 were imputed using these same methods. A categorized number of cigarettes smoked per day (numCIG) phenotype was used to assess smoking quantity. The numCIG was grouped as follows: 10 or less, 11–20, 21–30, 31 or more. All subjects included are classified as smokers, based on the criteria of having smoked at least 100 cigarettes in their lifetime. For each data set, the number of subjects with smoking information, sex distribution, mean ages of all participants, and the number of individuals in each numCIG category is shown in Table 2.

Statistical Analyses

Statistical analyses for animal studies were conducted using the StatView program. Data were analyzed by one-way or two-way analysis of variance with treatment or strain × treatment as the between subject factors. *P*-values <0.05 were considered significant, and were further assessed using the Neuman–Keuls *post hoc* test. For human association studies, statistical analyses were conducted using the PLINK program (Purcell *et al*, 2007). The numCIG was treated as a quantitative trait and analyzed by linear regression in each data set. In all association analyses, sex, and age were used as covariates, along with cancer diagnosis in the PLCO study. In the COGEND, COGA, and FSCD samples, a principal component covariate was also used to account for population stratification within these data sets. Additionally, in the COGA and FSCD samples where the primary phenotype is alcohol or cocaine dependence respectively, smokers may have comorbid alcohol or cocaine dependence, thus, drug affection status (case *vs* control for alcohol or cocaine dependence) was also used as a covariate in these data sets. Results from each individual study were used to conduct a meta-analysis, carried out using the GWAMA software (Magi and Morris, 2010). Cochrane's Q statistic *P*-values were calculated to measure between-study heterogeneity in the meta-analysis. Correction for multiple testing was applied to all significant results in each analysis using the Bonferonni correction (*P*-value × no. of markers); however, the uncorrected values are reported in the result tables. The number of data sets was not taken into account in the corrected *P*-values in the meta-analysis. Linkage disequilibrium maps were constructed using the Haploview program (Barrett *et al*, 2005).

Table 1 Chromosomal Position, Allele, Marker Location, and Minor Allele Frequencies (MAF) for the *GalR1* Gene Markers Analyzed in this Study

GalR1 marker	Chrom. position	Allele	Marker location	MAF (EA)	MAF (AA)
rs2850889	73087595	T/**C**	≈3 kb upstream	0.45	0.38
rs12953809	73087637	C/**T**	≈3 kb upstream	0.09	0.02
rs9959924	73096882	C/**T**	intron	0.09	0.35
rs2717164	73097544	T/**G**	intron	0.02	0.33
rs8097893	73112043	A/**G**	≈1 kb downstream	0.04	0.09
rs11877007	73112916	C/**T**	≈1 kb downstream	0.05	0.15
rs2156641	73114166	A/**C**	≈3 kb downstream	0.03	0.21

Abbreviations: AA, African-American; EA, European-American.
The minor allele is located on the right and bolded. MAF for both populations are based on frequencies from the MGS samples, which is the reference panel used for imputation in our study, and were determined using the Haploview program (Barrett *et al*, 2005).

Table 2 Number of Subjects with Smoking Information (N), Sex Distribution (Represented as Percentage), Mean Age, and the Number of Individuals in Each numCIG Category for All Studies Included

Study	*N*	Sex distribution (%)	Mean age (SD)	NumCIG
Molecular Genetics of Schizophrenia (EA)	1430	47.8% M; 52.2% F	50.6 (16.4)	⩽10 = 187
				11–20 = 359
				21–30 = 486
				⩾31 = 398
Molecular Genetics of Schizophrenia (AA)	487	39.2% M; 60.8% F	45.6 (13.1)	⩽10 = 86
				11–20 = 227
				21–30 = 129
				⩾31 = 45
Collaborative Genetic Study of Nicotine Dependence (EA)	1088	34.8% M; 65.2% F	37.1 (6.9)	⩽10 = 841
				11–20 = 122
				21–30 = 49
				⩾31 = 76
Collaborative Study on Genetics of Alcoholism (EA)	661	53.9% M; 46.1% F	42.8 (10.3)	⩽10 = 135
				11–20 = 294
				21–30 = 120
				⩾31 = 112
Family Study of Cocaine Dependence (EA)	307	51.2% M; 48.8% F	36.9 (8.8)	⩽10 = 61
				11–20 = 115
				21–30 = 49
				⩾31 = 82
Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trail (EA)	1469	61.8% M; 38.1% F	64.6 (6.7)	⩾10 = 324
				11–20 = 544
				21–30 = 368
				⩾31 = 233

Abbreviations: AA, African-American; EA, European-American; F, female; M, male.
Study names are listed on the left.

RESULTS

Galanin and GALR Expression in the VMB and NAc is Correlated with Nicotine Withdrawal Somatic Signs

In order to facilitate genetic correlation analyses for nicotine behaviors, we first characterized nicotine withdrawal in 25 of the BXD inbred strains. As depicted in Figure 1, nearly all BXD strains displayed an increase in somatic withdrawal signs following mecamylamine (2 mg/kg, s.c.) administration compared with saline treatment. There was a significant effect of both strain and treatment for mecamylamine-induced somatic signs ($F_{(26,47)} = 7.45$, $P < 0.001$ and $F_{(1,248)} = 76.27$, $P < 0.001$, respectively). The interaction between strain and treatment was also significant ($F_{(26,248)} = 2.08$, $P = 0.002$). The mouse strain distribution for this trait was continuous, characteristic of a quantitative trait driven by multiple genes, each constituting modest effect size (Brigman *et al*, 2009; Caspi and Moffitt, 2006). As scores following saline were very low, there was a very high genetic correlation between the strain mean pattern for mecamylamine-induced somatic signs and the difference in somatic signs scores between mecamylamine *vs* saline-treated animals (Pearson's $r = 0.911$, $P = 6.26E{-}14$, $n = 27$). The B6 and D2 progenitors did not exhibit extreme phenotypes, but variability for somatic signs across the BXD panel was still quite high. This variability is likely due to the interaction of progenitor alleles from multiple loci to cause an augmentation or diminution of the trait (Neumann *et al*, 1993; Mozhui *et al*,

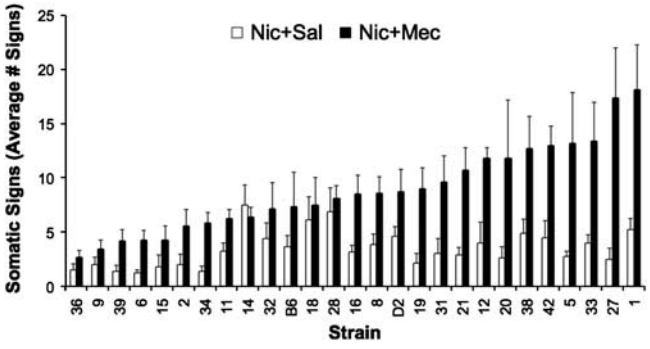

Figure 1 Distribution of mecamylamine-induced physical withdrawal signs in B6, D2, and BXD strains, characteristic of a quantitative trait. Following 7 days of nicotine infusion (24 mg/kg per day), mecamylamine (2 mg/kg, s.c.) precipitated somatic withdrawal signs in nearly all BXD RI strains. Each point represents the mean ± SEM of 6–8 mice per group.

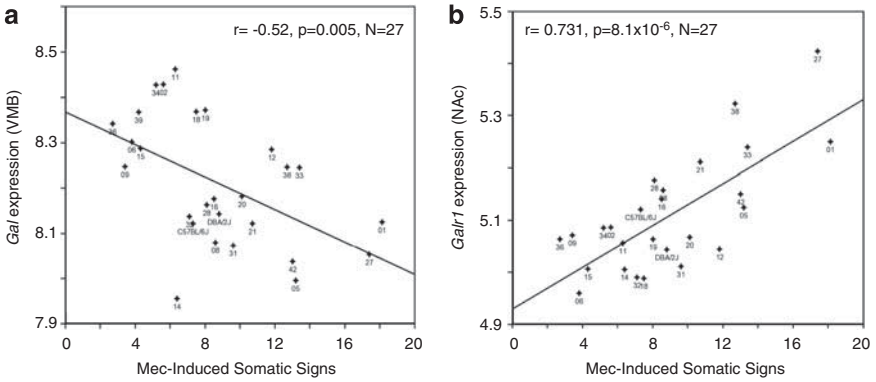

Figure 2 Correlation of galanin or GALR1 with somatic signs across BXD strains treated with chronic nicotine. Panels (a, b) depict expression correlations with mecamylamine-induced somatic signs. Panel (a) shows galanin expression in the ventral midbrain (VMB; Probeset 1460668_at in VCU BXD VTA Sal data set in GeneNetwork), and panel (b) displays GALR1 expression in the NAc (Probeset 1441329_at in VCU BXD NA Sal data set in GeneNetwork). *Y* axis values are $\log_2$ values of results from the RMA probe summarization and normalization method.

2007). As an initial screen to identify whether galanin signaling might influence nicotine behavioral traits, we utilized a genetic correlation analysis across the BXD inbred mouse panel. We used initial behavioral data to perform a genetic correlation between nicotine behavioral phenotypes and microarray expression data sets previously generated by our laboratory as well as other publicly available data sets. Using the GeneNetwork web resource for the study of genetics and genomics in complex traits (http://www.GeneNetwork.org), we screened nicotine withdrawal phenotypes for strong correlations with galanin or galanin receptor gene expression in whole brain, AMG, DST, NAc, HPC, and VMB expression databases. Mecamylamine-induced somatic signs, a physical measure of withdrawal, showed significant correlations with galanin expression in VMB (Figure 2a) as well as GALR1 expression in NAc (Figure 2b) across the BXD recombinant inbred strains. A difference score for somatic signs (Mec-saline) also showed strong correlations for *GalR1* expression in NAc, but correlation with *Gal* was no longer significant in VMB. Additional analyses in whole brain, AMG, DST, HPC, STR, and PFC were unremarkable for any significant correlation between mecamylamine-induced somatic signs and galanin or any galanin receptor (not shown).

Galnon Reduces Physical Signs of Withdrawal in the Mouse

ICR mice were chronically infused with nicotine (24 mg/kg per day) or saline for 7 days, and withdrawal signs were evaluated on day 8, 10 min after treatment with the nAChR antagonist, mecamylamine (2 mg/kg, s.c.). Mecamylamine precipitated a significant anxiety-like response, indicated by a decrease in the amount of time spent on the open arms of the plus maze, increased somatic signs, and a hyperalgesia response indicated by a decreased latency on the hotplate in nicotine-infused mice Pretreatment with galnon (0.5 mg/kg, i.p.) reversed expression of nicotine withdrawal-induced somatic signs ($F_{(3,16)} = 29.38$, $P < 0.001$) and hyperalgesia ($F_{(3,16)} = 7.64$, $P < 0.05$), but had no effect on anxiety-like behavior (Figure 3). Galnon alone did not produce significant effects in any withdrawal test in this paradigm.

Galnon Blocks the Reward-Like Effects of Nicotine as Measured in the CPP Test in Mice

To examine the role of GalRs in nicotine reward, mice were conditioned for 3 days with galnon at different doses before nicotine (0.5 mg/kg, s.c.) in the CPP paradigm. Nicotine induced a significant preference in mice, which was blocked by galnon pretreatment in a dose-dependent manner. As shown in Figure 4, nicotine CPP was significantly blocked by 0.02 mg/kg galnon ($F_{(5,49)} = 11.90$, $P < 0.05$) and 0.2 mg/kg galnon ($F_{(5,49)} = 11.90$, $P < 0.001$). Nicotine preference after 0.2 mg/kg galnon pretreatment did not differ from vehicle groups. In addition, we tested the effects of galnon on a lower dose of nicotine (0.1 mg/kg, s.c.). Galnon at 0.2 mg/kg did not enhance nicotine-induced preference at a low dose in the CPP test, but rather blocked it (vehicle/saline = 17 ± 12; vehicle/nicotine (0.1 mg/kg) = 49.8 ± 12.3*; galnon (0.2 mg/kg)/nicotine (0.1 mg/kg) = 8 ± 16; *$P < 0.05$). Galnon alone at the highest does used did not produce significant effects on locomotor activity or preference in this paradigm.

Variants in the *GALR1* Gene are Associated with Smoking Quantity

Association analyses were conducted in six data sets to assess the role of GALR1 in smoking quantity. We tested seven SNPs in the MGS European-American and African-American controls, and in European-American samples from the COGEND, COGA, FSCD, and PLCO data sets using the numCIG phenotype. The markers are either within the *GALR1* gene or within 4 kb of the gene. Results from these six studies were used to conduct a meta-analysis to evaluate the genetic association between GALR1 and smoking quantity, and increase the power of our statistical analyses. Results of the meta-analysis in Table 3 show a significant association with the markers rs9959924 and rs2717164 and smoking quantity. The marker rs9959924 remained significant after correction for multiple testing ($P = 0.01$). Beta coefficients reveal a protective effect for the variants. Linkage disequilibrium maps with r^2 values from the MGS European and African-American data sets are shown in

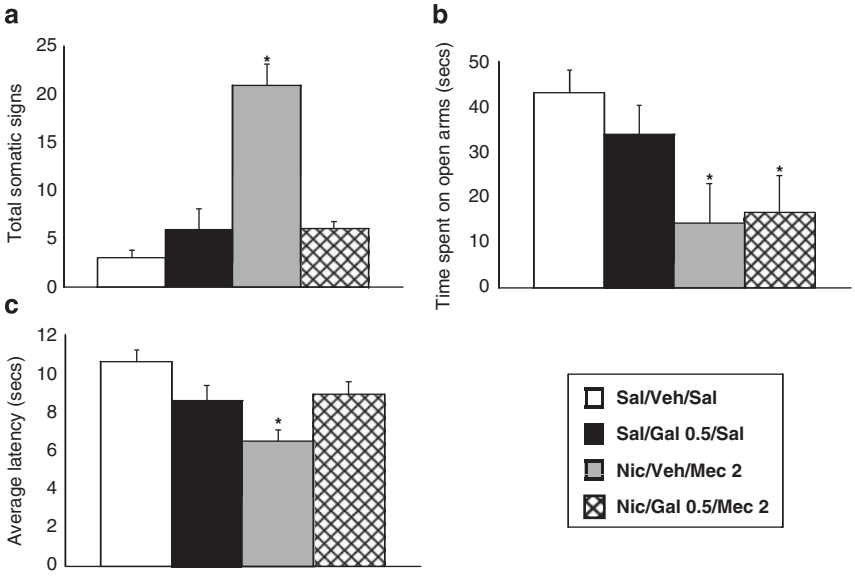

Figure 3 Galnon reverses mecamylamine-precipitated physical nicotine withdrawal signs in mice. In chronic nicotine-infused mice, mecamylamine (2 mg/kg, s.c.) administration precipitated a significant (a) anxiety-like response, (b) somatic signs, and (c) hyperalgesia response. The somatic signs and hyperalgesia response were reversed by pretreatment with galnon (0.5 mg/kg, i.p.). No effect of galnon was observed on anxiety-like behavior. Each point represents the mean ± SEM of six mice per group. $^*P<0.05$ *vs* control groups.

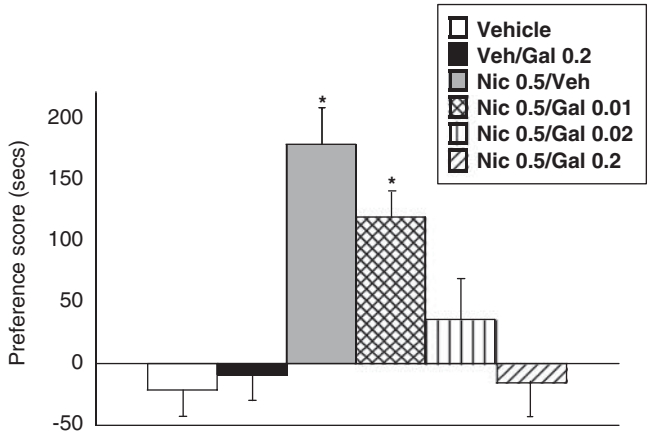

Figure 4 Galnon blocks nicotine reward using the CPP model in mice. Mice were conditioned for three days with nicotine (0.5 mg/kg, s.c.) and treated with the galanin agonist, galnon before nicotine to evaluate the role of galanin receptors in nicotine reward. Galnon (0.02 and 0.2 mg/kg, i.p) significantly blocked nicotine-induced preference in the CPP test. Each point represents the mean ± SEM of 8–10 mice per group. $^*P<0.05$ *vs* vehicle-treated groups.

Table 3 Meta-Analysis of the *GalR1* Gene Identifies Variants Associated with ND

	SNP	Meta-analysis ($n=5442$)		
		Allele	Beta	*P*-value
numCIG	rs2850889	C	0.01	0.60
	rs12953809	T	0.004	0.89
	rs9959924	T	−0.10	**0.001**
	rs2717164	G	−0.08	**0.02**
	rs8097893	G	−0.03	0.50
	rs11877007	T	−0.02	0.65
	rs2156641	C	−0.04	0.26

Meta-analysis was conducted using the MGS European and African-American samples, COGEND, COGA, FSCD, and PLCO studies. The number of cigarettes smoked per day (numCIG)/smoking quantity was used as a measure of ND. Cochran's Q statistic *P*-values were not significant for any marker tested ($P<0.05$), suggesting minimal heterogeneity between studies; therefore, the fixed effect *P*-values are reported in the table. Significant values, where $P<0.05$, are bolded.

Figure 5. The two significant markers are not in high linkage disequilibrium in either population ($r^2=0.17$ and 0.35 in European and African-American populations, respectively), suggesting the associations represent independent signals. Figure 6 represents a forest plot of the meta-analysis, which was constructed to show the effect size of the markers rs9959924 and rs2717164 in smoking quantity.

DISCUSSION

This study is among the first to demonstrate a role for galanin and GALR1 in ND. We found a strong correlation between basal expression of galanin and GALR1 and somatic signs of nicotine withdrawal across the BXD panel of inbred strains. In verification of these expression–behavioral correlations, we found that galnon, a galanin receptor agonist, blocks nicotine reward and reverses physical nicotine withdrawal signs in mice, suggesting a protective role for galanin and GALR1 in two important aspects of ND. Furthermore, genetic association data from our meta-analysis indicate a significant association with variants in the *GALR1* gene and smoking quantity, and support our animal behavioral studies, suggesting a protective effect in ND. Taken together, our studies support a protective role for galanin and GALR1 in ND.

Results from our genetic correlation analysis across the BXD inbred panel revealed significant correlations between

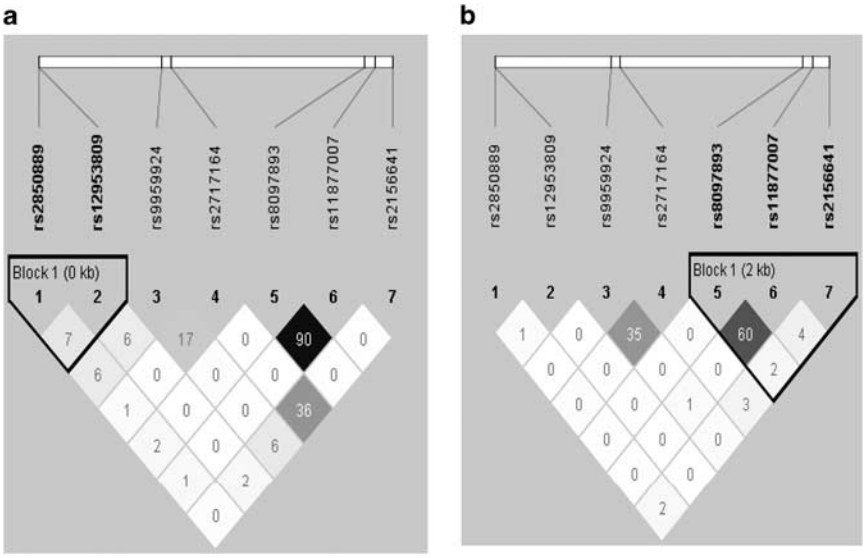

Figure 5 GALR1 linkage disequilibrium maps from the MGS control populations. Linkage disequilibrium maps showing r^2 values from the (a) MGS European-American and (b) MGS African-American control subjects. The two significant markers in the association study, rs9959924 and rs2717164, are not in high linkage disequilibrium in either population.

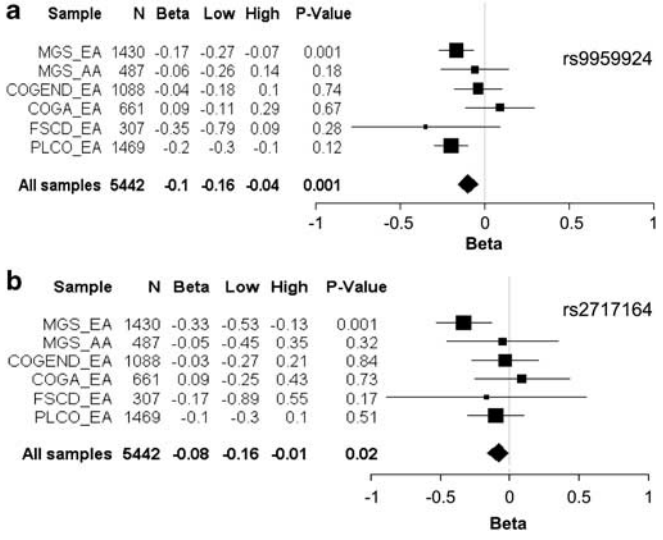

Figure 6 The *GALR1* gene is associated with a protective effect in ND. The results from six independent association studies (shown on the left) were used for a meta-analysis of the *GALR1* gene using the number of cigarettes/smoking quantity as a ND phenotype. Results show that the variants (a) rs9959924 and (b) rs2717164 are significantly associated with a protective effect in ND. In addition to sample study name, the number of individuals in each study (N), beta coefficients (Beta), upper and lower beta coefficient confidence intervals (Low, High), and *P*-values are also shown to the left of the plot. The area of each square in the plot is proportional to the study's weight in the meta-analysis. Horizontal lines represent confidence intervals. The *X* axis of the graph represents beta coefficients. The summary meta-analysis result is represented by the diamond. The points of the diamond do not overlap the line of no effect (vertical line shown at point 0) for either variant, suggesting an overall significant protective effect with rs9959924 and rs2717164 in ND. AA, African-American; EA, European-American.

nicotine withdrawal and galanin signaling within both the NAc and VMB. GALR1 expression in the NAc and galanin expression in the VMB were significantly correlated with mecamylamine-precipitated somatic signs. Further interrogation of galanin or GALR expression in whole brain, hippocampus, striatum, amygdala, and prefrontal cortex did not yield any significant correlations with nicotine withdrawal signs, suggesting region-specific effects of galanin and GALR expression on nicotine somatic withdrawal. Indeed, rats chronically infused with nicotine and subsequently administered mecamylamine site-specifically into the VTA, provide evidence that both the VTA and NAc have a role in nicotine withdrawal (Hildebrand *et al*, 1999). Higher basal GALR1 expression in the NAc was observed in strains with more severe nicotine withdrawal signs while lower galanin levels in VMB occurred in those strains. These findings are consistent with decreased galanin signaling contributing to more severe somatic withdrawal symptoms.

It is noted that the gene expression variance displayed in Figure 2 suggests that somewhat subtle (less than twofold) differences in galanin or GALR1 expression had relatively large effects on somatic signs of nicotine withdrawal. This might be due to the fact that the gene expression measurements were done on micro-dissected brain regions containing heterogeneous populations of cells. Thus, the microarrays might actually be detecting larger variance in galanin/GALR1 expression within a small group of cells, but that these differences are masked by 'stable' expression within surrounding tissue across the BXD strains. A similar phenomenon has been described and verified by *in situ* hybridization previously in studies on seizure-associated gene expression in hippocampus (Elliott *et al*, 2003). Additionally, the microarray results displayed here might not fully reflect actual differences in galanin or GALR1 protein abundance.

In the nicotine withdrawal behavioral paradigm, galnon reversed mecamylamine-precipitated nicotine withdrawal somatic signs in mice, as observed in previous behavioral studies on morphine withdrawal (Zachariou *et al*, 2003), and the hyperalgesia response. These results are consistent with our genetic correlation analysis showing an inverse

relationship between VMB galanin expression and somatic signs of nicotine withdrawal (Figure 2a). The inverse relationship between basal galanin expression in VMB and GALR1 expression in the NAc, is suggestive of adaptive changes within an agonist/receptor circuit. It will be important for future studies to determine the effect of chronic nicotine and nicotine withdrawal on galanin or GALR mRNA and protein expression in VMB and NAc to more fully characterize the role of galanin signaling in nicotine withdrawal.

Previous data also show that GALR1 mRNA levels are increased in the mouse LC after morphine withdrawal, a process thought to be an adaptive mechanism to counteract negative withdrawal effects (Zachariou *et al*, 2000). Our gene expression databases did not yet directly study the LC, thus, we could not assess a role of galanin signaling in that area for this study. Nicotine has been shown to increase the firing rate of noradrenergic neurons in the LC (Svensson and Engberg, 1980; Engberg and Hajos, 1994), and chronic nicotine binding to nicotinic receptors (nAChRs) on noradrenergic neurons in the LC stimulates norepinephrine release into limbic brain regions (Fu *et al*, 1998), an effect that produces aversive, stress-like states during drug withdrawal (Koob, 2008; Koob and Le Moal, 2008). As a result of the role of the LC in drug withdrawal states, and the actions of nicotine in this region, it will be of interest in future studies to examine galanin, and GALR1 expression in the LC after nicotine withdrawal.

It is noted that galnon had no effect in the plus maze test, an affective measure of nicotine withdrawal-induced anxiety-like behavior. Previous studies show that affective and physical nicotine withdrawal signs are mediated by different nAChR populations (Jackson *et al*, 2008). Specifically, the β4 nAChR subunit has been implicated in nicotine withdrawal somatic signs and hyperalgesia (Salas *et al*, 2004). It has been hypothesized that α3β2 and α3β4 nAChR subtypes are primarily involved in nicotine-induced norepinephrine release in the LC (Fu *et al*, 1998). Furthermore, noradrenergic neurons in the LC project to the VTA and NAc (Sara, 2009), brain regions in which we observed variance in basal galanin and GALR1 expression, respectively, correlating with nicotine withdrawal signs in the BXD strains. Thus, in the case of nicotine, it is possible that galanin is counteracting withdrawal effects mediated primarily by β4-containing nAChRs on noradrenergic neurons. On the basis of this, it is of interest in future studies to correlate galanin, GALR1, and β4 nAChR signaling and expression in various brain regions after nicotine withdrawal to validate this potential mechanism.

In the nicotine CPP paradigm, galnon attenuated the rewarding effects of nicotine in mice, suggesting a role for galanin receptors in the rewarding effects of nicotine. These data are consistent with previous reports implicating a protective role for galanin receptors in morphine and cocaine reward (Zachariou *et al*, 1999; Narasimhaiah *et al*, 2009). Galanin is reported to have an inhibitory effect on dopamine release in rat striatal slices (Tsuda *et al*, 1998), a finding that supports the ability of galnon to attenuate rewarding effects of drugs of abuse. Interestingly, a recent study found that Gal $^{-/-}$ mice are less sensitive to the rewarding effects of nicotine, as higher nicotine doses were required to produce a significant CPP in these mice compared with $^{+/+}$ counterparts (Neugebaur *et al*, 2011). This finding is the opposite of what would be expected, given our current results. These differences may be simply attributed to compensatory mechanisms in the Gal $^{-/-}$ mice and/or strain differences. Indeed, ICR mice were used in our studies *vs* Gal $^{+/+}$ mice bred on a 129 Ola/Hsd background in the Neugebaur *et al* study, and we observed a robust CPP at 0.5 mg/kg nicotine, while CPP was not observed in Gal $^{+/+}$ mice at doses higher than 0.18 mg/kg. On a more conceptual level, because galnon is a non-selective galanin receptor agonist, it is possible that stimulation of distinct galanin receptor subtypes in our study differentially modulates nicotine's actions in the CPP paradigm. Furthermore, it is conceivable that GalR subtypes activation by galanin (a non-selective agonist at the three GalR subtypes) modulate nicotine reward differentially, which could explain the results with the Gal $^{-/-}$ mice. Finally, it is possible that Gal exerts a direct activity through an allosteric mechanism on neuronal nicotinic receptors mediating nicotine reward such as α4β2* subtypes. Such interaction was reported for several endogenous peptides such as substance P, Lynx-1 and CGRP among others. Overall, these two studies suggest that Gal-mediated mechanisms and receptors probably modulate nicotine reward in a differential manner. It is necessary to further assess these possibilities through the use of subtype specific GalR $^{-/-}$ mice and pharmacological agents.

Our human genetic association data also implicate a role for the *GALR1* gene in ND. Our meta-analysis revealed that two variants within the *GALR1* gene were significantly associated with a protective effect against heavy smoking, based on the numCIG phenotype. The significant markers, rs959924 and rs2717164, are not in high linkage disequilibrium in either population tested, suggesting that the association represents two independent signals. Although the *P*-values in the human association studies were modest, the variant rs959924 remained significant after Bonferroni correction. These results are consistent with other human association studies, where the effects of individual variants are relatively small (Saccone *et al*, 2010). In essence, our human association analyses support the results obtained from gene expression and animal behavioral studies. These results also support a recent finding by Lori Am Tang *et al*, (2011), which showed that smokers attempting smoking cessation treatment with the minor allele of the *GALR1* gene variant rs2717162 have lower tobacco craving scores than individuals with the major and heterozygote alleles.

Although the effects of galnon, the agonist used in our behavioral studies, are mediated primarily through galanin receptors, and the non-peptide agonist is reported to have a higher affinity toward GALR1 (Sollenberg *et al*, 2005), it does not distinguish between GALR1-3 subtypes (Saar *et al*, 2002). Although all three receptors are expressed in brain areas implicated in drug dependence, including the VTA, substantia nigra, NAc, and LC (Mennicken *et al*, 2002), GALR1 is the predominant receptor expressed in the VTA, substantia nigra, and NAc, and consequently, is thought to have a predominant role in galanin-mediated regulation of dopamine neurotransmission (Hawes and Picciotto, 2004). To this end, GALR1 has been shown to couple to G-proteins, which decrease adenyl cyclase activity, thereby decreasing neuronal excitability (Wang *et al*, 1998),

implicating the GALR1 subtype as the most likely subtype responsible for decreased dopamine release (Tsuda *et al*, 1998). GALR1 mRNA levels are also increased in the mouse LC after morphine withdrawal (Zachariou *et al*, 2000). On the basis of these studies and our current results, we propose that the protective effects of galanin in ND are primarily mediated through GALR1; however, we cannot rule out the possibility that GALR2 and/or GALR3 contribute to the observed effects in our study, in particular, with the results of our CPP study.

Overall, the results of this study support a protective role for galanin and GALR1 in ND, and further suggest that galanin agonists may be a useful target for drug dependence therapy. These results also provide insight into protective genetic mechanisms of ND, and could prove to be beneficial in developing more individualized smoking cessation therapies.

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DISCLOSURE

The authors declare no conflict of interest.

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