Published in final edited form as:

Am J Med Genet B Neuropsychiatr Genet. 2016 December ; 171(8): 1057–1071. doi:10.1002/ajmg.b.32476.

Associations of rare nicotinic cholinergic receptor gene variants to nicotine and alcohol dependence

Lingjun Zuo^{1,*}, Yunlong Tan², Chiang-Shan R. Li¹, Zhiren Wang², Kesheng Wang³, Xiangyang Zhang⁴, Xiandong Lin⁵, Xiangning Chen⁶, Chunlong Zhong⁷, Xiaoping Wang⁸, Xiaoyun Guo^{1,9}, Jijun Wang⁹, Lu Lu^{10,11}, and Xingguang Luo^{1,2,*}

¹Department of Psychiatry, Yale University School of Medicine, New Haven, CT, USA

²Biological Psychiatry Research Center, Beijing Huilongguan Hospital, Beijing, China

³Department of Biostatistics and Epidemiology, College of Public Health, East Tennessee State University, Johnson City, TN, USA

⁴Department of Psychiatry and Behavioral Sciences, The University of Texas Health Science Center at Houston, Houston, TX, USA

⁵Provincial Key Laboratory of Translational Cancer Medicine, Fujian Provincial Cancer Hospital, Fuzhou, Fujian, China

⁶Nevada Institute of Personalized Medicine and Department of Psychology, University of Nevada, Las Vegas, NV, USA

⁷Department of Neurosurgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

⁸Department of Neurology, Shanghai First People's Hospital, Shanghai Jiao-Tong University, Shanghai, China

⁹Department of EEG & Neuroimaging, Shanghai Mental Health Center, Shanghai, China

¹⁰Provincial Key Laboratory for Inflammation and Molecular Drug Target, Medical College of Nantong University, China

¹¹Departments of Genetics, Genomics, Informatics, Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN, USA

Abstract

Objective—Nicotine's rewarding effects are mediated through distinct subunits of nAChRs, encoded by different nicotinic cholinergic receptor (*CHRN*) genes and expressed in discrete regions in the brain. In the present study, we aimed to test the associations between rare variants at *CHRN* genes and nicotine dependence (ND) and alcohol dependence (AD).

*Corresponding authors: Xingguang Luo and Lingjun Zuo; 950 Campbell Avenue, West Haven, CT 06516. Xingguang.Luo@yale.edu or Lingjun.Zuo@yale.edu; Tel: 203-932-5711.

Conflict of interest: The authors have declared that no competing interests exist.

Methods—A total of 26,498 subjects with 9 different neuropsychiatric disorders in 15 independent cohorts, which were genotyped on Illumina, Affymetrix or PERLEGEN microarray platforms, were analyzed. Associations between rare variants (minor allele frequency (MAF) < 0.05) at *CHRN* genes and nicotine dependence and alcohol dependence were tested. The mRNA expression of all *Chrn* genes in whole mouse brain and ten specific brain areas was investigated.

Results—All *CHRN* genes except the muscle-type *CHRNA1*, including eight genomic regions containing 11 neuronal *CHRN* genes and three genomic regions containing four muscle-type *CHRN* genes, were significantly associated with ND and/or AD. All of these genes were expressed in the mouse brain.

Conclusion—We conclude that *CHRN*s are associated with ND (mainly) and AD, supporting the hypothesis that the full catalog of ND/AD risk genes may contain most neuronal nAChRs-encoding genes.

Keywords

CHRN; nAChR; nicotine dependence; alcohol dependence; rare variant; mRNA expression

Introduction

There are two major subtypes of acetylcholine receptors (AChR) — metabotropic muscarinic AChRs and ionotopic nicotinic AChRs (nAChRs). Both are activated by the endogenous neurotransmitter acetylcholine. The nAChRs can be either neuronal or muscle-type. The former are heavily expressed in central and peripheral nervous systems and in some non-neuronal tissues such as the adrenal medulla. In contrast, the muscle-type receptor is localized primarily at neuromuscular junctions. nAChRs are pentameric ligand-gated ion channels composed of membrane-spanning subunits arranged around a cation channel. [Lindstrom 2003] Each subunit is encoded by a single nicotinic cholinergic receptor (*CHRN*) gene. Neuronal nAChR subunits include $\alpha 2$ through $\alpha 10$ and $\beta 2$ through $\beta 4$ that are encoded by *CHRNA*s 2 through 10 and *CHRNA*s 2 through 4, respectively (Supplementary Table S1). The muscle-type nAChR subunits include $\alpha 1$, $\beta 1$, γ , δ , and ϵ that are encoded by *CHRNA1*, *B1*, *G*, *D* and *E*, respectively [Gotti and others 2007] (Supplementary Table S1). nAChRs are typically heteropentamers composed of α - and β -subunits in different ratios or homopentamers of α subunits. Two main classes of neuronal nAChRs are known: α -bungarotoxin (α Bgtx)-sensitive receptors composed of heteromeric $\alpha 7$ – $\alpha 10$ -subunits or a homomeric form (e.g., ($\alpha 7$)₅); and α Bgtx-insensitive receptors composed of heteromeric $\alpha 2$ – $\alpha 6$ and $\beta 2$ – $\beta 4$ -subunits and binding nicotine but not α Bgtx (such as $\alpha 4\beta 2^*$ and $\alpha 3\beta 4^*$ receptors, where * indicates other subunits). Homopentameric receptors such as ($\alpha 7$)₅ nAChRs have five identical acetylcholine-binding sites in each molecule, whereas the heteropentameric receptors have only two. In each heteromeric receptor, two α subunits ($\alpha 2$ – $\alpha 4$ or $\alpha 6$) carry the principal acetylcholine-binding sites. The two non- α subunits ($\beta 2$ or $\beta 4$) carry complementary components of the binding sites. The fifth accessory subunit ($\alpha 5$, $\beta 2$, $\beta 3$, or $\beta 4$) does not participate in binding.

Distinct neuronal nAChRs are distributed in different parts of the CNS and contribute to the psychoactive properties of nicotine and to the etiology of many neuropsychiatric illnesses.

Here we focused on the associations of nAChRs with nicotine dependence (ND) and alcohol dependence (AD). Although the ND- or AD- related traits have been assessed using different approaches in numerous genetic association studies, they have moderate agreement among them. Variants in most of the *CHRN* gene regions have been related to ND-related traits (Supplementary Table S2) [Chen and others 2013; Cui and others 2013; Fowler and others 2011; Greenbaum and others 2009; Keskitalo-Vuokko and others 2011; Lou and others 2006; Pergadia and others 2009; Philibert and others 2009; Saccone and others 2010; Stephens and others 2013; Thorgeirsson and others 2008; Thorgeirsson and others 2010; Wang and others 2014; Xie and others 2011; Yang and others 2014]. The majority of studies have focused on the *CHRNA5-A3-B4* cluster [Berrettini and others 2008; Bierut and others 2007; Bierut and others 2008; Caporaso and others 2009; Fowler and others 2011; Greenbaum and others 2009; Hartz and others 2012; Li 2008; Liu and others 2010; Saccone and others 2009; Saccone and others 2010; Saccone and others 2007a; Stephens and others 2013; Thorgeirsson and others 2008; Thorgeirsson and others 2010; Tobacco and Genetics Consortium 2010; Wang and others 2009a] which is most reliably associated with ND-related traits and the association has been verified by many functional studies. Some associations at this locus were highly significant with p values as low as 10^{-73} . Additionally, a large body of neurobiological and genetic evidence supports an important role for the *CHRNA7* gene in ND [Greenbaum and others 2006; Saccone and others 2010]. Several other genome-wide association studies (GWASs) identified association peaks for ND at *CHRNA4* [Bierut and others 2007; Wei and others 2012], which has been replicated in the same and numerous other studies [Bierut and others 2007; Feng and others 2004; Li and others 2005; Saccone and others 2009; Saccone and others 2010; Wei and others 2012]. Many GWASs demonstrated an association of *CHRNA3-CHRNA6* with ND [Bierut and others 2007; Saccone and others 2007a; Saccone and others 2007b; Thorgeirsson and others 2010], and the findings were replicated by candidate gene studies [Culverhouse and others 2014; Hoft and others 2009; Saccone and others 2009; Saccone and others 2010; Wei and others 2012; Won and others 2014]. Evidence also supports involvement of seven other *CHRN* loci (except for *CHRNA2*) in genetic susceptibility to ND, including neuronal *CHRNA2* [Bergen and others 1999; Faraone and others 2004; Heitjan and others 2008; Swan and others 2006; Yang and others 2014], *CHRNA9* [Ehringer and others 2010; Yang and others 2014], *CHRNA10* [Pergadia and others 2009; Saccone and others 2010], *CHRNA2* [Conti and others 2008; Perkins and others 2009] and muscle-type *CHRNA1*, *CHRNA1*, *CHRNA1* [Philibert and others 2009; Saccone and others 2009; Saccone and others 2010] and *CHRNA1* [Keskitalo-Vuokko and others 2011; Saccone and others 2009; Saccone and others 2010]. Finally, *CHRNA5-A3-B4* [Wang and others 2009b], *CHRNA7* [Kamens and others 2010], *CHRNA4* [Coon and others 2014], *CHRNA3-A6* [Haller and others 2014] and *CHRNA2* [Ehringer and others 2007] have also been associated to AD or alcohol use related traits (Supplementary Table S2), although these associations were much weaker than those with ND.

An important discovery in human genetics is that many diseases appear to be caused by constellations of multiple rare, regionally concentrated variants, rather than by common variants [Dickson and others 2010]. The combined effects of rare variants distributed across the genome may be highly significant. The main goal of the present study is to test whether

or not rare variants in *CHRN*s are associated with both ND and AD. We are also interested in possible contributions of *CHRN* variants to schizophrenia [Lin and others 2014], depression [Philip and others 2010], Alzheimer's disease [Lombardo and Maskos 2015], Parkinson's disease [Burghaus and others 2003], amyotrophic lateral sclerosis [Sabatelli and others 2009] and ischemic stroke [Liu and others 2014].

Materials and Methods

Subjects

We initially evaluated a total of 27 independent cohorts with different neuropsychiatric disorders as available from the database of Genotypes and Phenotypes (dbGaP). In the present study, we only analyzed case-control data, and excluded six family-based datasets. Six other datasets with absolute genomic inflation factor (λ) larger than 1.1 as derived from QQ plots were also excluded (data not shown). Consequently, 15 datasets as listed in Supplementary Table S3 were included in the analyses. These 15 independent cohorts included a total of 26,498 subjects with 9 different neuropsychiatric disorders, genotyped on Illumina, Affymetrix or PERLEGEN microarray platforms. These 9 disorders included ND, AD, major depression, schizophrenia, Alzheimer's disease, ALS, early onset stroke, ischemic stroke and Parkinson's disease. Detailed demographics data are shown in Supplementary Table S3.

These subjects contained four cohorts with ND or AD: (1) COGEND+UW-TTUTC (EA) cohort included 836 European-American cases with ND (15.4% were AD), and 80 European-American controls (17.8% were AD); (2) COGEND+UW-TTUTC (AA) cohort included 392 African-American cases with ND (7.5% were AD), and 79 African-American controls (18.6% were AD); (3) SAGE+COGA (EA) cohort included 1,409 European-American cases with AD (58% were ND) and 1,518 European-American controls; and (4) SAGE+COGA (AA) cohort included 681 African-American cases with AD (66% were ND) and 508 African-American controls. Affected subjects with ND were defined by a commonly used definition of ND – a current score of 4 or more (out of a maximum score of 10) on the Fagerström Test for Nicotine Dependence (FTND). Control status was defined as an individual who smoked at least 100 cigarettes during their lifetime, yet never became dependent (lifetime FTND=0). Affected subjects with AD met DSM-IV criteria for AD [American Psychiatric Association 1994] and controls were defined as individuals who had been exposed to alcohol (and possibly to other drugs), but had never become dependent on these substances. Additionally, all cases and controls in these four cohorts were screened to exclude individuals with major axis I disorders, including schizophrenia, mood disorders, and anxiety disorders. This study was approved by the Institutional Review Board of Yale University.

Imputation and data cleaning

To make the genetic marker sets consistent across different cohorts, we imputed the untyped SNPs across each *CHRN* gene region (detailed in Supplementary Methods). We stringently cleaned the phenotype and genotype data within each ethnicity prior to association analysis (detailed in Supplementary Methods).

Association tests for individual rare variants

A total of 179–429 (in subjects of European descent) and 136–620 (in subjects of African descent) cleaned rare SNPs with $0 < \text{MAF} < 0.05$ in controls were extracted for association analysis (Table 1). In each cohort, the allele frequencies of each SNP were compared between cases and controls using logistic regression as implemented in PLINK [Purcell and others 2007]. Diagnosis and alleles each served as the dependent and independent variables, with sex, age and the first 10 principal components of ancestries as covariates. In non-AD and non-ND cohorts, “alcohol drinking” and “smoking”, if available, were included as additional covariates in the models. Different cohorts were analyzed independently. The MAFs and the minimal p values of the most significant risk SNPs and the numbers of the nominally-significant risk SNPs ($p < 0.05$) in all cohorts are shown in Table 1. An α was set to guard against type I error with Bonferroni correction. The false discovery rate (q value) for each SNP was estimated from the p values within each cohort using the R package QVALUE [Storey and Tibshirani 2003].

Association tests for family-wide rare variant constellations with diseases

Sixteen *CHRN* genes in 12 regions (Supplementary Table S1) have similar sequences and the subunits encoded by these genes have similar structures and might share similar biological features. Five subunits comprise one nACh receptor; that is, five subunits integrate to exert the receptor function. Thus, global synthetic effects of rare variant constellations across the whole family of *CHRN*s might exist. We tested these effects using a score-type program, SCORE-Seq [Lin and Tang 2011]. The mutation information was aggregated by virtue of a weighted linear combination across all rare variants of all *CHRN* genes/regions, and then related to each disease phenotype using regression models. Sex, age and the first 10 principal components of ancestries, as estimated using the program EIGENSTRAT [Price and others 2006], served as the covariates in the regression models. The first 10 principal components explained more than 95% of genetic variance in our samples. In non-AD and non-ND cohorts, “alcohol drinking” and “smoking”, if available, were included as additional covariates in the models.

We first analyzed the associations between diseases and rare variant constellations where the rare variants came with $0 < \text{MAF} < 0.01$ (T1 test). If it was significant, we then tested those rare variants with $0 < \text{MAF} < 0.05$ (T5 test) to validate the T1 test results. We also employed the F_p test to validate the findings: (1) In the T1 and T5 tests, the MAF upper bound thresholds were fixed at 0.01 and 0.05, respectively, and the weight was fixed at 1; and (2) In the F_p tests, the MAF upper bound threshold was fixed at 0.05, and the weight was set as $1/\sqrt{p(1-p)}$ where p was the estimated MAF with pseudo counts in the pooled sample. Statistical significance was assessed using a bootstrap procedure with 1 million times of resampling [Lin and Tang 2011] and corrected for the number of effective datasets ($n=15$). All association analyses were performed within the same ethnicity.

Association tests for single-locus rare variant constellations with diseases

After significant associations between family-wide rare variant constellations and diseases in some cohorts were identified, we decomposed these significant associations to identify the significant single-locus rare variant constellations across each gene region. The same

analytic procedure was performed as the above and the results were examined with statistical correction for the number of gene regions ($n=12$). Finally, we meta-analyzed the two datasets with ND, two datasets with AD, and two sub-datasets with ND+AD, respectively, using the program MetaP (<http://igm.cumc.columbia.edu/MetaP/metap.php>).

Bioinformatics analysis

To explore the potential functions of the individual rare variants included in the rare variant constellations, we predicted their functions using a series of bioinformatics analyses. We used UCSC Genome Browser data or other bioinformatics analysis software packages (e.g., FuncPred [Xu and Taylor 2009] or VEP [McLaren and others 2010]) to see whether the risk SNPs are located within methylated CpG islands, within copy number variations (CNVs) or in exonic splicing silencers (ESS) or enhancers (ESE). Additionally, Polyphen [Adzhubei and others 2013] and SIFT [Ng and Henikoff 2003] were applied to predict the pathogenicity in order to see whether these rare variants affect protein function or structure. The conservation of these SNPs across 17 species was also predicted [King and others 2005].

Detection of *CHRN* mRNA expression in mouse brains

The mRNA expression of all *Chrn* genes in whole mouse brain and ten specific brain areas was investigated (detailed in Supplementary Methods).

Results

No individual rare variant was significantly associated with diseases (Table 1)

Among the rare variants, 0–26 variants were each nominally associated with diseases in different cohorts ($p < 0.05$). Minimal p value in each cohort was between 5.4×10^{-4} and 0.134. However, after Bonferroni correction, no variants were significantly associated with diseases ($p > \alpha$). The q values for all SNPs were > 0.05 .

There were significant associations between family-wide rare variant constellations and ND and AD in four cohorts (Table 2)

There are no significant associations between global rare variant constellations across the whole family of *CHRN* gene regions and major depression, schizophrenia, Alzheimer's disease, amyotrophic lateral sclerosis, early onset stroke, ischemic stroke and Parkinson's disease ($p > 0.05/15 = 3.3 \times 10^{-3}$).

However, there are significant associations between these family-wide rare variant constellations and ND in EAs and AAs, and AD in AAs (1.5×10^{-58} p 7.9×10^{-4}). The T5 and Fp tests validated the T1 test very well, with more significant results than the latter.

Although there was no significant association between family-wide rare variant constellations and AD in EAs, there was significant association with nicotine and alcohol co-dependence (ND+AD) in the subset of this EA sample (p values: T1= 1.6×10^{-8} ; T5= 8.6×10^{-17} and Fp= 1.1×10^{-17}).

We found that although family-wide rare variant constellations were associated with AD comorbid with ND (e.g., 1.7×10^{-6} p 7.9×10^{-4} in AAs that included 66% ND, and 1.1×10^{-17} p 1.6×10^{-8} for AD+ND in EAs that was ND too), associations were much more significantly associated with ND-alone (e.g., 1.9×10^{-35} p 1.3×10^{-4} for ND in AAs, and 1.5×10^{-58} p 5.0×10^{-18} for ND in EAs).

There were significant associations between eleven single-locus rare variant constellations across each CHRN gene/region and ND or AD in the above four cohorts (Tables 3 and S4)

CHRNA10 ($T1=7.5 \times 10^{-3}$, $T5=9.1 \times 10^{-6}$ and $Fp=1.4 \times 10^{-6}$), *CHRNA7* ($T1=4.5 \times 10^{-3}$, $T5=5.1 \times 10^{-4}$, and $Fp=4.0 \times 10^{-4}$) and *CHRNA9* ($T1=0.039$, $T5=4.0 \times 10^{-6}$ and $Fp=3.5 \times 10^{-6}$) were nominally significantly associated with AD in AAs. Similarly, *CHRNA4*, *CHRNA5-CHRNA3-CHRNA4*, *CHRNA7*, *CHRNA9* and *CHRNA5-CHRNA3-CHRNA4* were nominally significantly associated with AD+ND in EAs. *CHRNA1*, *CHRNA5-CHRNA3-CHRNA4* and *CHRNA7* were nominally significantly associated with ND in AAs. *CHRNA10*, *CHRNA2*, *CHRNA4*, *CHRNA5-CHRNA3-CHRNA4*, *CHRNA7*, *CHRNA9*, *CHRNA3-CHRNA6* and *CHRNA6* were nominally significantly associated with ND in EAs. Among these associations, *CHRNA7* was nominally significantly and replicably associated with ND, ND+AD or AD across four cohorts (Table 3). All of these associations remained to be nominally significant in the meta-analysis of EAs and AAs (Table S4). After correction for 12 regions ($\alpha=4.2 \times 10^{-3}$ for T1 test), *CHRNA5-CHRNA3-CHRNA4* and *CHRNA5-CHRNA3-CHRNA4* were significantly associated with AD+ND in EAs; *CHRNA5-CHRNA3-CHRNA4* was significantly associated with ND in AAs; *CHRNA10*, *CHRNA2*, *CHRNA5-CHRNA3-CHRNA4*, *CHRNA7*, *CHRNA3-CHRNA6* and *CHRNA6* were significantly associated with ND in EAs. All of these associations remained significant in the meta-analysis of EAs and AAs (Table S4). Among these associations, *CHRNA5-CHRNA3-CHRNA4* was significantly and replicably associated with ND in EAs and AAs and with AD+ND in EAs. The T5 and Fp tests for these associations were all highly significant and verified the T1 test.

Bioinformatics analysis (Table 4)

Almost all variants with potential functions were missense variants located in exons. A total of 14 potentially functional SNPs were identified (Table 4). Among them, rs76927517 at *CHRNA1* is located within a 538bp CpG island, whose methylation status may affect the expression of *CHRNA1*. Five SNPs are located in exonic splicing silencers or enhancers and thus might influence the splicing activity. rs2231547 at *CHRNA10* and rs12914008 at *CHRNA4* are predicted to be conservative across species. Six SNPs are predicted to be damaging and five SNPs are predicted to be deleterious, suggesting that they may significantly affect the protein function or structure. Finally, rs79835149 at *CHRNA5* may result in a premature stop codon, leading to a shortened transcript.

All Chrn genes were expressed in mouse brain, in distinct areas and at different levels (Table 5)

We found that all *Chrn* genes were expressed in mouse brain at different levels. All of these genes were expressed in the hippocampus, in which the genes with most highly abundant expression [standardized expression value (SEV) > 9] were *Chrna7* (SEV=10.88), *Chrn2*

(SEV=10.22) and *Chrna4* (SEV=9.95). These three were also modestly to highly abundantly expressed in other brain areas examined (SEV=7.03–11.15), with 1.0–17.8-fold changes (FC) in mRNA level compared to the expression base, among which *Chrna7* was most abundantly expressed in the hippocampus (SEV=10.88) and *Chrn2* and *Chrna4* were most abundant in the hypothalamus (SEV=11.15 and 11.09, respectively).

Chrna5, *Chrna3* and *Chrn4* were expressed in multiple brain areas (SEV=7.22–9.35), with a 1.2–5.1-fold change in mRNA expression levels compared to the expression base (SEV=7). *Chrna6* and *Chrn3* were expressed in several areas (SEV=7.05–10.37), among which *a6* was abundantly expressed in the midbrain (FC=10.4) and *Chrn3* abundantly in the pituitary (FC=4.1). *Chrna2* was modestly to abundantly expressed in multiple areas (SEV=7.14–8.87) with 1.1–3.7-fold changes too. Both *Chrna9* and *Chrna10* were modestly expressed in the brain (SEV<8) that included the cortex. Interestingly, the muscle-type *Chrn* genes including *Chrna1*, *Chrn1*, *Chrn4*, *Chrne* and *Chrn5* were expressed at a low level in most brain areas (SEV<8), whereas *Chrn1* in the pituitary, *Chrn4* in the whole brain, ventral tegmental area (VTA) and nucleus accumbens (NAc), *Chrne* in the whole brain and *Chrn5* in the hippocampus and cerebellum were expressed at abundant levels (SEV=8.00–9.15).

Discussion

In the present study, we investigated a total of 16 *CHRN* genes in 12 genomic regions, including 11 neuronal *CHRN* genes in 8 genomic regions and 5 muscle-type *CHRN* genes in 4 genomic regions (Supplementary Table S1). We found that all *CHRN* genes but the muscle-type *CHRN1* were significantly associated with ND and/or AD. Further, all of these genes were expressed in the mouse brain, which supports previous findings and hypothesis that the development of ND and AD involves more than one nAChR subunits.

Nicotine's psychoactive effects appear to be mediated through distinct subunits of nAChRs, encoded by different genes and expressed in discrete regions in the brain [Lindstrom 2003]. Adult midbrain neurons express a variety of nAChR subunit mRNAs, including $\alpha 2$, 4, 5, 6, 7, 9, $\beta 2$ and $\beta 4$, as confirmed by this study, although the $\alpha 5$ expression in the midbrain is very low in our study (SEV=6.6; data not shown). Because the midbrain, including the VTA, is widely implicated in drug dependence [Subramaniyan and Dani 2015], these receptor subunits are all plausible candidates for a functional role in mediating nicotine effects on addiction. For instance, a large portfolio of different nAChR subtypes expressed in the VTA may define dopaminergic neuronal subpopulations with different implications for the reinforcing effects of nicotine [Faure and others 2014]. In addition, the number of binding sites per pentamer varies from two to five, depending on its composition, and the non-equivalent binding sites formed by different subunits have different affinities for agonists and antagonists. For example, five functional nAChR subtypes in dopaminergic terminals in the striatum have different affinities for nicotine; that is, $\alpha 4\alpha 6\beta 2\beta 3$, $\alpha 6\beta 2\beta 3$ and $\alpha 6\beta 2$ have the highest sensitivity to nicotine whereas $\alpha 4\beta 2$ and $\alpha 4\beta 2\alpha 5$ have lower affinity for nicotine [Champtiaux and others 2002; Drenan and others 2008; Grady and others 2007]. The expression of $\alpha 2$, 4 and 7 and $\beta 1$, 2 and 4 in mouse striatum was confirmed by our study.

The most robust associations, which were highly significant (even after correction for multiple comparisons) and replicable, were found between *CHRNA5-A3-B4* and ND/AD, consistent with a previous report [Greenbaum and Lerer 2009]. We found that this gene region was significantly associated with ND in EAs and AAs, and with AD+ND in EAs, suggesting shared ND and AD susceptibility on this region. In support, variants in this gene cluster have been reported to influence and promote the early use of tobacco and alcohol [Schlaepfer and others 2008]. Further, a partial agonist (CP-601932) for the $\alpha 3\beta 4^*$ nAChRs has been used as smoking cessation therapy and may have utility too in decreasing alcohol consumption and seeking in cigarette smokers [Chatterjee and others 2011; McKee and others 2009].

CHRNA5 and *CHRNA3* are in high linkage disequilibrium (LD), but they are in lower LD with *CHRNA4* [Berrettini and others 2008]. In humans, mRNA encoding the $\alpha 5$ subunit has been detected in the nervous system (including the cortex) but appears to be mainly expressed in the cerebellum, medial habenula, and the autonomic ganglia [Flora and others 2000]. In particular, $\alpha 4\beta 2\alpha 5$ -containing receptors are expressed on dopaminergic neurons in rat and mouse striatum and are involved in nicotine-elicited dopamine release [Salminen and others 2004; Zoli and others 2002]. In addition, $\alpha 5$ -containing receptors are also expressed on GABAergic neurons in the striatum and VTA. $\alpha 3$ -containing nAChRs (mainly $\alpha Bgtx$ -insensitive $\alpha 3\beta 4^*$ receptor) were found in several regions of the rodent brain, including the VTA, pineal gland, medial habenula, interpeduncular nucleus (IPN), brainstem nuclei, hippocampus and cerebellum [Gahring and others 2004; Perry and others 2002; Turner and Kellar 2005]. Both *CHRNA3* and *CHRNA5* genes are expressed in the neural circuits of reward processing and learning as well as cognitive and affective control, involving the NAc, entorhinal cortex, cerebellum and amygdala. Our study confirmed that these two genes were expressed in the hippocampus, VTA, NAc, cerebellum, amygdala, hypothalamus and cortex in mice. Unfortunately, we did not examine the expression of *CHRNA5*, *CHRNA3* and *CHRNA4* in medial habenula where these genes were reported to be highly expressed. Many of these brain regions in humans are implicated in the etiological process of ND. For instance, nicotine activates the habenulo-interpeduncular pathway through $\alpha 5$ -containing nAChRs, which can trigger an inhibitory motivational signal that acts to limit nicotine intake [Fowler and others 2011]. Future research combining optical imaging and mouse genetic models may significantly advance our understanding of the roles of these genes and the molecular mechanisms underlying ND [Pistillo and others 2015].

The nicotinic $\beta 4$ -subunit is thought to be widely expressed in the mouse peripheral nerve system, with a restricted expression pattern in the central nervous system (CNS). In CNS, it was previously observed at high levels only in the medial habenula and IPN [Klink and others 2001; Quick and others 1999; Salas and others 2004]. Functional data have shown changes in nicotine intake following manipulations of $\beta 4$ in medial habenula, supporting its role in the reinforcing effect of nicotine [Rose 2007]. It was observed at relatively low densities in the rat and mouse substantia nigra and VTA [Klink and others 2001; Salas and others 2003] but not in NAc, cerebellum or cortex, in accord with our findings.

$\alpha 3$ is a principal subunit that could harbor a binding site. $\alpha 5$ and $\beta 4$ subunits do not have acetylcholine binding sites and cannot form functional nAChRs by themselves. They may

contribute to receptor targeting and localization in the neuronal membrane, but do not play a direct role in receptor–ligand binding, suggesting their auxiliary roles when expressed with other α - and β -subunits in conferring risk for ND.

Another robust association, which was highly significant and replicable across all four cohorts, was also found between *CHRNA7* and ND/AD. We found that this gene was significantly associated with ND both in EAs and AAs, with ND+AD in EAs, and with AD in AAs. ND and AD share rare risk variant constellations at *CHRNA7*. The homomeric $\alpha 7$ -receptors were previously reported to be widely expressed within the CNS [Brody and others 2006], including the VTA, prefrontal and motor cortex, basal ganglia, hippocampus, cerebellum, and lateral and medial geniculate bodies, and with lower expression in the thalamus [Conejero-Goldberg and others 2008]. Upregulation of $\alpha 7$ -subunit containing nAChRs after chronic nicotine treatment has been reported in mouse striatum, midbrain and cortex [Nuutinen and others 2005; Pakkanen and others 2005], in some but not all studies. Our findings confirmed that $\alpha 7$ was abundant in the hippocampus, midbrain, VTA, NAc, cerebellum, striatum, amygdala, hypothalamus, pituitary and cortex. The hippocampus and neocortex are involved in learning, memory and emotion regulation [Gotti and others 2007; Klink and others 2001] and the VTA–NAc pathway is critical in drug-induced rewards [Benowitz 2008], supporting a role of $\alpha 7$ in ND and AD.

We found that *CHRNA4* was significantly associated with ND and ND+AD in EAs. The $\alpha 4$ subunit usually combines the $\beta 2$ subunit to form a neuronal $\alpha 4\beta 2^*$ nAChR. This receptor is α Bgtx-insensitive and the predominant receptor subtype in the brain of humans and other mammalian species. Unlike that the expression pattern of other subunits is much more restricted to particular brain regions [Gotti and Clementi 2004; Gotti and others 2006; Wilens and Decker 2007], the $\alpha 4\beta 2^*$ receptor is widely expressed within the CNS, [Brody and others 2006] including the mesolimbic dopamine system and the VTA–NAc pathway. We confirmed in mice that both $\alpha 4$ and $\beta 2$ were expressed across all eleven brain regions examined and were highly abundant in hippocampus, midbrain, amygdala, hypothalamus, pituitary and cortex (SEV>9). Previous evidence suggested highest expression of $\alpha 4$, and $\beta 2$ in the thalamus, [Picciotto and others 1998] which we unfortunately did not examine in this study. The $\alpha 4$ is a principal subunit with an acetylcholine binding site; thus, the $\alpha 4\beta 2^*$ receptor has high affinity for nicotine [Hogg and others 2003] and is sufficient to mediate nicotine reward, tolerance and sensitization [Tapper and others 2004]. This receptor is upregulated in the brains of smokers after chronic nicotine exposure [Breese and others 1997]. The $\alpha 4\beta 2^*$ receptor may co-assemble with additional units to form more complex subtypes such as $(\alpha 4\beta 2)2\alpha 3$, $(\alpha 4\beta 2)2\alpha 6$, $(\alpha 4\beta 2)2\alpha 5$ or $(\alpha 4\beta 2)2\beta 3$ [Gotti and others 2006; Quik and others 2000]. Different $\alpha 4/\beta 2$ ratios in the receptor can lead to different pharmacological and functional properties. For example, $(\alpha 4\beta 2)2\alpha 5$ acetylcholine receptor may play a role in conferring the aversive experience of nicotine use, limiting the drive to smoke.

CHRNA6–*CHRNA3* was significantly associated with ND in EAs. Long-term self-administration of nicotine has been found to upregulate $\alpha 6$ - and $\beta 3$ -containing receptors in rat brain [Parker and others 2004]. The $\alpha 6$ - and $\beta 3$ -subunit upregulation is of special interest because these subunits are co-expressed as $\alpha 6\beta 3^*$ or $\alpha 4\alpha 6\beta 2\beta 3$ nAChRs [Champtiaux

and others 2003; Gotti and others 2007; Salminen and others 2007] in midbrain dopamine neurons and modulate dopamine release [Champtiaux and others 2002; Exley and others 2008; Meyer and others 2008]. In rat and mouse striatum, $\alpha 6\beta 2^*$ and $\alpha 4(\text{non-}\alpha 6)\beta 2^*$ are the major nAChR subtype populations, differentially expressed by dopaminergic and non-dopaminergic neurons and involved in mediating dopamine release [Champtiaux and others 2003; Gotti and others 2005; Le Novere and others 1996; Zoli and others 2002]. An auxiliary subunit, $\beta 3$ assembles with other neuronal nAChR α - and β -subunits, and supports the stability and transport of $\alpha 6^*$ nAChRs in dopaminergic neurons [Gaimarri and others 2007; Gotti and others 2005]. $\beta 3$ -containing nAChRs have a physiologically significant role in dopaminergic neurotransmission and many nAChRs expressed on mice substantia nigra-VTA dopaminergic neurons are $\beta 3$ -dependent [Cui and others 2003]. $\alpha 4\alpha 6\beta 2\beta 3$ receptor possesses the highest affinity for nicotine among all nicotinic receptor subtypes reported thus far [Champtiaux and others 2002; Drenan and others 2008; Grady and others 2007], and may play an important role in regulating dopamine-related behaviors, including mediating the reinforcing effects of nicotine. Furthermore, $\alpha 6$ and $\beta 3$ may also co-express in the VTA [Moretti and others 2004], as confirmed by our study.

Of lesser statistical strength were the findings of three other neuronal nAChR loci (*CHRNA2*, *A9* and *A10*) in association with ND. *CHRNA2* was significantly associated with ND in EAs. $\alpha 2$ -mRNA was reported to be expressed in the midbrain as well as in the cerebral cortex [Ishii and others 2005]. $\alpha 2$ -containing receptors were highly expressed in monkeys and humans, suggesting that the expression of this subunit in the brain is species specific. We found that $\alpha 2$ was expressed in midbrain, hippocampus, amygdala, striatum and cortex. Many studies have demonstrated that this is a functional gene for nicotine response [Lotfipour and others 2013; Picciotto and others 1998]. Some functional rare variants have been identified in this gene [Dash and Li 2014; Dash and others 2014]. Nicotine influences the local balance between neuronal excitation and inhibition, gates long-term potentiation, and directs information flow through the hippocampal circuits via the activation of $\alpha 2^*$ nAChRs, providing a cellular basis of nicotine-mediated cognitive enhancement [Nakauchi and others 2007]. Additionally, we found that both *CHRNA9* and *CHRNA10* were significantly associated with ND in EAs and with AD in AAs. *CHRNA9* was also significantly associated with ND+AD in EAs. ND and AD share risk rare variant constellations at the *CHRNA9* and *CHRNA10*. $\alpha 9$ -subunit was reported to be partially expressed in the pituitary [Lips and others 2002], which was confirmed by our study. We also found $\alpha 9$ in hippocampus, midbrain, cerebellum, amygdala, hypothalamus and cortex and $\alpha 10$ in hippocampus, amygdala and cortex, but not in other brain regions, consistent with previous reports [Gotti and others 2006]. Functionally, $\alpha 9$ subunit can form a homopentamer or heterooligomeric cation-selective channels in conjunction with nAChR $\alpha 10$ [Sgard and others 2002], which suggests the potential of *CHRNA9* as a functional gene.

Five nicotinic receptor subunits encoded by *CHRNA1*, *CHRNB1*, *CHRNA9*, *CHRNA10* and *CHRNA11* are abundantly expressed in the neuromuscular junctions but not in the brain, according to earlier reports. However, we detected these subunits in all ten mouse brain regions but the midbrain in this study, consistent with the findings that *CHRNA1*, *CHRNA9*, *CHRNA10* and *CHRNA11* were associated with ND/ND+AD, and earlier findings that *CHRNA1*, *CHRNA9*, *CHRNA10* and *CHRNA11* were associated with ND, as discussed above.

On the other hand, these associations in our study were identified only in one cohort but not replicated in others. For example, *CHRNA1* was significantly associated with ND in AAs; *CHRNE* was significantly associated with ND in EAs; and *CHRNA7/CHRNA9* was significantly associated with ND+AD in EAs. Thus, these findings and their significance need to be evaluated again in the future.

We did not find significant association of *CHRNA2* (neuronal nAChR) with ND/AD in the T1 test, even in the meta-analysis ($p > 0.05$; Tables 3 and S4). However, in the T5 and Fp tests, it is significantly associated with ND/AD across four cohorts ($p < \alpha$; Table 3) and in the meta-analysis (Table S4). Further, we found that $\beta 2$ was highly expressed in all brain regions examined, which may explain the important role of $\beta 2$ in the ND-related nAChRs as presented in the above.

In summary, we found all *Chrn* genes were expressed in mouse brain and most *CHRN* gene regions were involved in ND/AD. First, except for the muscle-type *CHRNA1*, variants in almost all other 11 nAChR subunit-encoding gene regions (containing 15 genes) were associated with ND, among which, *CHRNA7*, *CHRNA9* and *CHRNA10* were also associated with AD. Second, although ND and AD shared some susceptibility genes, associations with ND are much stronger than AD. Although there was no significant association between family-wide rare variant constellations and AD in an EA sample, there was significant association with ND+AD in the subset of this EA sample. While the sample size of this subset became smaller, the association became stronger, which is probably because *CHRN*s are mainly associated with ND rather than AD. Third, associations between ND/AD and both *CHRNA5-A3-B4* and *CHRNA7* are the most robust in that they were significant, replicable across more than three cohorts, and consistent with previous reports and expression findings. Fourth, many findings were highly significant with p values as low as 10^{-39} (for T5 and Fp tests). When multiple rare variants are significantly associated with ND, rare variant constellation analysis highlights their cumulating effects. The more positive variants are included, the more significant the associations of the constellations would be. This is why associations by T5 test were much more significant than T1 test and why the p values for T5 test could be so small. Fifth, bioinformatics analysis indicates that a number of potentially functional individual SNPs might underlie the risk rare variant constellations. They might influence the methylation, splicing or shortening of the risk genes; or they might alter the protein function or structure directly. We conclude that *CHRN*s are associated with ND (mainly) and AD, consistent with previous findings and the hypothesis that “the full catalog of ND genes may contain all the neuronal nAChRs-encoding genes, each responsible for a particular intermediate phenotype, such as reinforcement, withdrawal, tolerance, and sensitization” [Greenbaum and Lerer 2009].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank for Drs. Picciotto, Basu and Williams' helpful comments. This work was supported in part by National Institute on Drug Abuse (NIDA) grant K01DA029643 and K02DA026990, National Institute on Alcohol Abuse and

Alcoholism (NIAAA) grants R21AA021380, R21AA020319, R21AA023237, U01AA13499, U01AA016662 and U01AA016667, the National Alliance for Research on Schizophrenia and Depression (NARSAD) Award 17616 (L.Z.) and ABMRF/The Foundation for Alcohol Research (L.Z.). We thank NIH GWAS Data Repository, the Contributing Investigator(s) who contributed the phenotype and genotype data from his/her original study (e.g., Drs. Bierut, Edenberg, Heath, Singleton, Hardy, Foroud, Myers, Gejman, Faraone, Sonuga-Barke, Sullivan, Nurnberger, Devlin, Monaco, etc.), and the primary funding organization that supported the contributing study. Funding and other supports for phenotype and genotype data were provided through the National Institutes of Health (NIH) Genes, Environment and Health Initiative (GEI) (U01HG004422, U01HG004436 and U01HG004438); the GENEVA Coordinating Center (U01HG004446); the NIAAA (U10AA008401, R01AA013320, P60AA011998); the NIDA (R01DA013423); the National Cancer Institute (P01 CA089392); the Division of Neuroscience, the NIA National Institute of Neurological Disorders and Stroke (NINDS); the NINDS Human Genetics Resource Center DNA and Cell Line Repository; the NIH contract "High throughput genotyping for studying the genetic contributions to human disease" (HHSN268200782096C); the Center for Inherited Disease Research (CIDR); a Cooperative Agreement with the Division of Adult and Community Health, Centers for Disease Control and Prevention; the NIH Office of Research on Women's Health (ORWH) (R01NS45012); the Department of Veterans Affairs; the University of Maryland General Clinical Research Center (M01RR165001), the National Center for Research Resources, NIH; the National Institute of Mental Health (R01MH059160, R01MH59565, R01MH59566, R01MH59571, R01MH59586, R01MH59587, R01MH59588, R01MH60870, R01MH60879, R01MH61675, R01MH62873, R01MH081803, R01MH67257, R01MH81800, U01MH46276, U01MH46282, U01MH46289, U01MH46318, U01MH79469, U01MH79470 and R01MH67257); the NIMH Genetics Initiative for Bipolar Disorder; the Genetic Association Information Network (GAIN); the Genetic Consortium for Late Onset Alzheimer's Disease; the Autism Genome Project, the MARC: Risk Mechanisms in Alcoholism and Comorbidity; the Molecular Genetics of Schizophrenia Collaboration; the Medical Research Council (G0601030) and the Wellcome Trust (075491/Z/04), University of Oxford; the Netherlands Scientific Organization (904-61-090, 904-61-193, 480-04-004, 400-05-717, NWO Genomics, SPI 56-464-1419) the Centre for Neurogenomics and Cognitive Research (CNCR-VU); Netherlands Study of Depression and Anxiety (NESDA) and the Netherlands Twin Register (NTR); and the European Union (EU/WLRT-2001-01254), ZonMW (geestkracht program, 10-000-1002). Assistance with phenotype harmonization and genotype cleaning, as well as with general study coordination, was provided by the Genetic Consortium for Late Onset Alzheimer's Disease, the GENEVA Coordinating Center (U01 HG004446), and the National Center for Biotechnology Information. Genotyping was performed at the Johns Hopkins University Center for Inherited Disease Research, and GlaxoSmithKline, R&D Limited. The datasets used for the analyses described in this manuscript were obtained from dbGaP at <http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gap>. XL had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The financial sponsors had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

References

- Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet*. 2013; Chapter 7(Unit7):20.
- American Psychiatric Association. Diagnostic and statistical manual of mental disorders. Washington, DC: American Psychiatric Press; 1994.
- Benowitz NL. Neurobiology of nicotine addiction: implications for smoking cessation treatment. *Am J Med*. 2008; 121(4 Suppl 1):S3–10.
- Bergen AW, Korszak JF, Weissbecker KA, Goldstein AM. A genome-wide search for loci contributing to smoking and alcoholism. *Genet Epidemiol*. 1999; 17(Suppl 1):S55–60. [PubMed: 10597412]
- Berrettini W, Yuan X, Tozzi F, Song K, Francks C, Chilcoat H, Waterworth D, Muglia P, Mooser V. Alpha-5/alpha-3 nicotinic receptor subunit alleles increase risk for heavy smoking. *Mol Psychiatry*. 2008; 13(4):368–373. [PubMed: 18227835]
- Bierut LJ, Madden PA, Breslau N, Johnson EO, Hatsukami D, Pomerleau OF, Swan GE, Rutter J, Bertelsen S, Fox L, Fugman D, Goate AM, Hinrichs AL, Konvicka K, Martin NG, Montgomery GW, Saccone NL, Saccone SF, Wang JC, Chase GA, Rice JP, Ballinger DG. Novel genes identified in a high-density genome wide association study for nicotine dependence. *Hum Mol Genet*. 2007; 16(1):24–35. [PubMed: 17158188]
- Bierut LJ, Stitzel JA, Wang JC, Hinrichs AL, Grucza RA, Xuei X, Saccone NL, Saccone SF, Bertelsen S, Fox L, Horton WJ, Breslau N, Budde J, Cloninger CR, Dick DM, Foroud T, Hatsukami D, Hesselbrock V, Johnson EO, Kramer J, Kuperman S, Madden PA, Mayo K, Nurnberger J Jr, Pomerleau O, Porjesz B, Reyes O, Schuckit M, Swan G, Tischfield JA, Edenberg HJ, Rice JP, Goate AM. Variants in nicotinic receptors and risk for nicotine dependence. *Am J Psychiatry*. 2008; 165(9):1163–1171. [PubMed: 18519524]

- Breese CR, Marks MJ, Logel J, Adams CE, Sullivan B, Collins AC, Leonard S. Effect of smoking history on [3H]nicotine binding in human postmortem brain. *The Journal of pharmacology and experimental therapeutics*. 1997; 282(1):7–13. [PubMed: 9223534]
- Brody AL, Mandelkern MA, London ED, Olmstead RE, Farahi J, Scheibal D, Jou J, Allen V, Tionson E, Chefer SI, Koren AO, Mukhin AG. Cigarette smoking saturates brain alpha 4 beta 2 nicotinic acetylcholine receptors. *Arch Gen Psychiatry*. 2006; 63(8):907–915. [PubMed: 16894067]
- Burghaus L, Schutz U, Krempel U, Lindstrom J, Schroder H. Loss of nicotinic acetylcholine receptor subunits alpha4 and alpha7 in the cerebral cortex of Parkinson patients. *Parkinsonism Relat Disord*. 2003; 9(5):243–246. [PubMed: 12781587]
- Caporaso N, Gu F, Chatterjee N, Sheng-Chih J, Yu K, Yeager M, Chen C, Jacobs K, Wheeler W, Landi MT, Ziegler RG, Hunter DJ, Chanock S, Hankinson S, Kraft P, Bergen AW. Genome-wide and candidate gene association study of cigarette smoking behaviors. *PLoS One*. 2009; 4(2):e4653. [PubMed: 19247474]
- Champtiaux N, Gotti C, Cordero-Erausquin M, David DJ, Przybylski C, Lena C, Clementi F, Moretti M, Rossi FM, Le Novère N, McIntosh JM, Gardier AM, Changeux JP. Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J Neurosci*. 2003; 23(21):7820–7829. [PubMed: 12944511]
- Champtiaux N, Han ZY, Bessis A, Rossi FM, Zoli M, Marubio L, McIntosh JM, Changeux JP. Distribution and pharmacology of alpha 6-containing nicotinic acetylcholine receptors analyzed with mutant mice. *J Neurosci*. 2002; 22(4):1208–1217. [PubMed: 11850448]
- Chatterjee S, Steensland P, Simms JA, Holgate J, Coe JW, Hurst RS, Shaffer CL, Lowe J, Rollema H, Bartlett SE. Partial agonists of the alpha3beta4* neuronal nicotinic acetylcholine receptor reduce ethanol consumption and seeking in rats. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2011; 36(3):603–615. [PubMed: 21048701]
- Chen HI, Shinkai T, Utsunomiya K, Yamada K, Sakata S, Fukunaka Y, Hwang R, De Luca V, Ohmori O, Kennedy JL, Chuang HY, Nakamura J. Possible association of nicotinic acetylcholine receptor gene (CHRNA4 and CHRNA2) polymorphisms with nicotine dependence in Japanese males: an exploratory study. *Pharmacopsychiatry*. 2013; 46(2):77–82. [PubMed: 23037950]
- Conejero-Goldberg C, Davies P, Ulloa L. Alpha7 nicotinic acetylcholine receptor: a link between inflammation and neurodegeneration. *Neurosci Biobehav Rev*. 2008; 32(4):693–706. [PubMed: 18180036]
- Conti DV, Lee W, Li D, Liu J, Van Den Berg D, Thomas PD, Bergen AW, Swan GE, Tyndale RF, Benowitz NL, Lerman C. Nicotinic acetylcholine receptor beta2 subunit gene implicated in a systems-based candidate gene study of smoking cessation. *Hum Mol Genet*. 2008; 17(18):2834–2848. [PubMed: 18593715]
- Coon H, Piasecki TM, Cook EH, Dunn D, Mermelstein RJ, Weiss RB, Cannon DS. Association of the CHRNA4 neuronal nicotinic receptor subunit gene with frequency of binge drinking in young adults. *Alcohol Clin Exp Res*. 2014; 38(4):930–937. [PubMed: 24428733]
- Cui C, Booker TK, Allen RS, Grady SR, Whiteaker P, Marks MJ, Salminen O, Tritto T, Butt CM, Allen WR, Stitzel JA, McIntosh JM, Boulter J, Collins AC, Heinemann SF. The beta3 nicotinic receptor subunit: a component of alpha-conotoxin MII-binding nicotinic acetylcholine receptors that modulate dopamine release and related behaviors. *J Neurosci*. 2003; 23(35):11045–11053. [PubMed: 14657161]
- Cui WY, Wang S, Yang J, Yi SG, Yoon D, Kim YJ, Payne TJ, Ma JZ, Park T, Li MD. Significant association of CHRNA3 variants with nicotine dependence in multiple ethnic populations. *Mol Psychiatry*. 2013; 18(11):1149–1151. [PubMed: 23319001]
- Culverhouse RC, Johnson EO, Breslau N, Hatsukami DK, Sadler B, Brooks AI, Hesselbrock VM, Schuckit MA, Tischfield JA, Goate AM, Saccone NL, Bierut LJ. Multiple distinct CHRNA3-CHRNA6 variants are genetic risk factors for nicotine dependence in African Americans and European Americans. *Addiction*. 2014; 109(5):814–822. [PubMed: 24401102]
- Dash B, Li MD. Two rare variations, D478N and D478E, that occur at the same amino acid residue in nicotinic acetylcholine receptor (nAChR) alpha2 subunit influence nAChR function. *Neuropharmacology*. 2014; 85:471–481. [PubMed: 24950454]

- Dash B, Lukas RJ, Li MD. A signal peptide missense mutation associated with nicotine dependence alters alpha2*-nicotinic acetylcholine receptor function. *Neuropharmacology*. 2014; 79:715–725. [PubMed: 24467848]
- Dickson SP, Wang K, Krantz I, Hakonarson H, Goldstein DB. Rare variants create synthetic genome-wide associations. *PLoS Biol*. 2010; 8(1):e1000294. [PubMed: 20126254]
- Drenan RM, Grady SR, Whiteaker P, McClure-Begley T, McKinney S, Miwa JM, Bupp S, Heintz N, McIntosh JM, Bencherif M, Marks MJ, Lester HA. In vivo activation of midbrain dopamine neurons via sensitized, high-affinity alpha 6 nicotinic acetylcholine receptors. *Neuron*. 2008; 60(1):123–136. [PubMed: 18940593]
- Ehringer MA, Clegg HV, Collins AC, Corley RP, Crowley T, Hewitt JK, Hopfer CJ, Krauter K, Lessem J, Rhee SH, Schlaepfer I, Smolen A, Stallings MC, Young SE, Zeiger JS. Association of the neuronal nicotinic receptor beta2 subunit gene (CHRNA2) with subjective responses to alcohol and nicotine. *Am J Med Genet B Neuropsychiatr Genet*. 2007; 144B(5):596–604. [PubMed: 17226798]
- Ehringer MA, McQueen MB, Hoft NR, Saccone NL, Stitzel JA, Wang JC, Bierut LJ. Association of CHRNA2 genes with “dizziness” to tobacco. *Am J Med Genet B Neuropsychiatr Genet*. 2010; 153B(2):600–609. [PubMed: 19760673]
- Exley R, Clements MA, Hartung H, McIntosh JM, Cragg SJ. Alpha6-containing nicotinic acetylcholine receptors dominate the nicotine control of dopamine neurotransmission in nucleus accumbens. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2008; 33(9):2158–2166. [PubMed: 18033235]
- Faraone SV, Su J, Taylor L, Wilcox M, Van Eerdewegh P, Tsuang MT. A novel permutation testing method implicates sixteen nicotinic acetylcholine receptor genes as risk factors for smoking in schizophrenia families. *Hum Hered*. 2004; 57(2):59–68. [PubMed: 15192278]
- Faure P, Tolu S, Valverde S, Naude J. Role of nicotinic acetylcholine receptors in regulating dopamine neuron activity. *Neuroscience*. 2014; 282C:86–100.
- Feng Y, Niu T, Xing H, Xu X, Chen C, Peng S, Wang L, Laird N. A common haplotype of the nicotine acetylcholine receptor alpha 4 subunit gene is associated with vulnerability to nicotine addiction in men. *Am J Hum Genet*. 2004; 75(1):112–121. [PubMed: 15154117]
- Flora A, Schulz R, Benfante R, Battaglioli E, Terzano S, Clementi F, Fornasari D. Neuronal and extraneuronal expression and regulation of the human alpha5 nicotinic receptor subunit gene. *J Neurochem*. 2000; 75(1):18–27. [PubMed: 10854242]
- Fowler CD, Lu Q, Johnson PM, Marks MJ, Kenny PJ. Habenular alpha5 nicotinic receptor subunit signalling controls nicotine intake. *Nature*. 2011; 471(7340):597–601. [PubMed: 21278726]
- Gahring LC, Persiyanov K, Dunn D, Weiss R, Meyer EL, Rogers SW. Mouse strain-specific nicotinic acetylcholine receptor expression by inhibitory interneurons and astrocytes in the dorsal hippocampus. *J Comp Neurol*. 2004; 468(3):334–346. [PubMed: 14681929]
- Gaimarri A, Moretti M, Riganti L, Zanardi A, Clementi F, Gotti C. Regulation of neuronal nicotinic receptor traffic and expression. *Brain Res Rev*. 2007; 55(1):134–143. [PubMed: 17383007]
- Gotti C, Clementi F. Neuronal nicotinic receptors: from structure to pathology. *Progress in neurobiology*. 2004; 74(6):363–396. [PubMed: 15649582]
- Gotti C, Moretti M, Clementi F, Riganti L, McIntosh JM, Collins AC, Marks MJ, Whiteaker P. Expression of nigrostriatal alpha 6-containing nicotinic acetylcholine receptors is selectively reduced, but not eliminated, by beta 3 subunit gene deletion. *Molecular pharmacology*. 2005; 67(6):2007–2015. [PubMed: 15749993]
- Gotti C, Moretti M, Gaimarri A, Zanardi A, Clementi F, Zoli M. Heterogeneity and complexity of native brain nicotinic receptors. *Biochemical pharmacology*. 2007; 74(8):1102–1111. [PubMed: 17597586]
- Gotti C, Zoli M, Clementi F. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol Sci*. 2006; 27(9):482–491. [PubMed: 16876883]
- Grady SR, Salminen O, Laverty DC, Whiteaker P, McIntosh JM, Collins AC, Marks MJ. The subtypes of nicotinic acetylcholine receptors on dopaminergic terminals of mouse striatum. *Biochemical pharmacology*. 2007; 74(8):1235–1246. [PubMed: 17825262]

- Greenbaum L, Kanyas K, Karni O, Merbl Y, Olender T, Horowitz A, Yakir A, Lancet D, Ben-Asher E, Lerer B. Why do young women smoke? I. Direct and interactive effects of environment, psychological characteristics and nicotinic cholinergic receptor genes. *Mol Psychiatry*. 2006; 11(3):312–322. 223. [PubMed: 16314871]
- Greenbaum L, Lerer B. Differential contribution of genetic variation in multiple brain nicotinic cholinergic receptors to nicotine dependence: recent progress and emerging open questions. *Mol Psychiatry*. 2009; 14(10):912–945. [PubMed: 19564872]
- Greenbaum L, Rigbi A, Teltsh O, Lerer B. Role of genetic variants in the CHRNA5-CHRNA3-CHRNA4 cluster in nicotine dependence risk: importance of gene-environment interplay. *Mol Psychiatry*. 2009; 14(9):828–830.
- Haller G, Kapoor M, Budde J, Xuei X, Edenberg H, Nurnberger J, Kramer J, Brooks A, Tischfield J, Almasy L, Agrawal A, Bucholz K, Rice J, Saccone N, Bierut L, Goate A. Rare missense variants in CHRNA3 and CHRNA5 are associated with risk of alcohol and cocaine dependence. *Hum Mol Genet*. 2014; 23(3):810–819. [PubMed: 24057674]
- Hartz SM, Short SE, Saccone NL, Culverhouse R, Chen L, Schwantes-An TH, Coon H, Han Y, Stephens SH, Sun J, Chen X, Ducci F, Dueker N, Franceschini N, Frank J, Geller F, Gubjartsson D, Hansel NN, Jiang C, Keskitalo-Vuokko K, Liu Z, Lyytikainen LP, Michel M, Rawal R, Rosenberger A, Scheet P, Shaffer JR, Teumer A, Thompson JR, Vink JM, Vogelzangs N, Wenzlaff AS, Wheeler W, Xiao X, Yang BZ, Aggen SH, Balmforth AJ, Baumeister SE, Beaty T, Bennett S, Bergen AW, Boyd HA, Broms U, Campbell H, Chatterjee N, Chen J, Cheng YC, Cichon S, Couper D, Cucca F, Dick DM, Foroud T, Furberg H, Giegling I, Gu F, Hall AS, Hallfors J, Han S, Hartmann AM, Hayward C, Heikkila K, Hewitt JK, Hottenga JJ, Jensen MK, Jousilahti P, Kaakinen M, Kittner SJ, Konte B, Korhonen T, Landi MT, Laatikainen T, Leppert M, Levy SM, Mathias RA, McNeil DW, Medland SE, Montgomery GW, Muley T, Murray T, Nauck M, North K, Pergadia M, Polasek O, Ramos EM, Ripatti S, Risch A, Ruczinski I, Rudan I, Salomaa V, Schlessinger D, Styrkarsdottir U, Terracciano A, Uda M, Willemsen G, Wu X, Abecasis G, Barnes K, Bickeboller H, Boerwinkle E, Boomsma DI, Caporaso N, Duan J, Edenberg HJ, Francks C, Gejman PV, Gelernter J, Grabe HJ, Hops H, Jarvelin MR, Viikari J, Kahonen M, Kendler KS, Lehtimäki T, Levinson DF, Marazita ML, Marchini J, Melbye M, Mitchell BD, Murray JC, Nothen MM, Penninx BW, Raitakari O, Rietschel M, Rujescu D, Samani NJ, Sanders AR, Schwartz AG, Shete S, Shi J, Spitz M, Stefansson K, Swan GE, Thorgeirsson T, Volzke H, Wei Q, Wichmann HE, Amos CI, Breslau N, Cannon DS, Ehringer M, Grucza R, Hatsukami D, Heath A, Johnson EO, Kaprio J, Madden P, Martin NG, Stevens VL, Stitzel JA, Weiss RB, Kraft P, Bierut LJ. Increased genetic vulnerability to smoking at CHRNA5 in early-onset smokers. *Arch Gen Psychiatry*. 2012; 69(8):854–860. [PubMed: 22868939]
- Heitjan DF, Guo M, Ray R, Wileyto EP, Epstein LH, Lerman C. Identification of pharmacogenetic markers in smoking cessation therapy. *Am J Med Genet B Neuropsychiatr Genet*. 2008; 147B(6): 712–719. [PubMed: 18165968]
- Hoft NR, Corley RP, McQueen MB, Schlaepfer IR, Huizinga D, Ehringer MA. Genetic association of the CHRNA6 and CHRNA3 genes with tobacco dependence in a nationally representative sample. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2009; 34(3):698–706. [PubMed: 18704094]
- Hogg RC, Raggenbass M, Bertrand D. Nicotinic acetylcholine receptors: from structure to brain function. *Rev Physiol Biochem Pharmacol*. 2003; 147:1–46. [PubMed: 12783266]
- Ishii K, Wong JK, Sumikawa K. Comparison of alpha2 nicotinic acetylcholine receptor subunit mRNA expression in the central nervous system of rats and mice. *J Comp Neurol*. 2005; 493(2):241–260. [PubMed: 16255031]
- Kamens HM, Andersen J, Picciotto MR. Modulation of ethanol consumption by genetic and pharmacological manipulation of nicotinic acetylcholine receptors in mice. *Psychopharmacology (Berl)*. 2010; 208(4):613–626. [PubMed: 20072781]
- Keskitalo-Vuokko K, Pitkaniemi J, Broms U, Heliövaara M, Aromaa A, Perola M, Ripatti S, Salminen O, Salomaa V, Loukola A, Kaprio J. Associations of nicotine intake measures with CHRNA genes in Finnish smokers. *Nicotine Tob Res*. 2011; 13(8):686–690. [PubMed: 21498873]

- King DC, Taylor J, Elnitski L, Chiaromonte F, Miller W, Hardison RC. Evaluation of regulatory potential and conservation scores for detecting cis-regulatory modules in aligned mammalian genome sequences. *Genome research*. 2005; 15(8):1051–1060. [PubMed: 16024817]
- Klink R, de Kerchove d'Exaerde A, Zoli M, Changeux JP. Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. *J Neurosci*. 2001; 21(5):1452–1463. [PubMed: 11222635]
- Le Novère N, Zoli M, Changeux JP. Neuronal nicotinic receptor alpha 6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. *Eur J Neurosci*. 1996; 8(11):2428–2439. [PubMed: 8950106]
- Li MD. Identifying susceptibility loci for nicotine dependence: 2008 update based on recent genome-wide linkage analyses. *Hum Genet*. 2008; 123(2):119–131. [PubMed: 18205015]
- Li MD, Beuten J, Ma JZ, Payne TJ, Lou XY, Garcia V, Duenes AS, Crews KM, Elston RC. Ethnic- and gender-specific association of the nicotinic acetylcholine receptor alpha4 subunit gene (CHRNA4) with nicotine dependence. *Hum Mol Genet*. 2005; 14(9):1211–1219. [PubMed: 15790597]
- Lin DY, Tang ZZ. A general framework for detecting disease associations with rare variants in sequencing studies. *Am J Hum Genet*. 2011; 89(3):354–367. [PubMed: 21885029]
- Lin H, Hsu FC, Baumann BH, Coulter DA, Anderson SA, Lynch DR. Cortical parvalbumin GABAergic deficits with alpha7 nicotinic acetylcholine receptor deletion: implications for schizophrenia. *Mol Cell Neurosci*. 2014; 61:163–175. [PubMed: 24983521]
- Lindstrom JM. Nicotinic acetylcholine receptors of muscles and nerves: comparison of their structures, functional roles, and vulnerability to pathology. *Annals of the New York Academy of Sciences*. 2003; 998:41–52. [PubMed: 14592862]
- Lips KS, Pfeil U, Kummer W. Coexpression of alpha 9 and alpha 10 nicotinic acetylcholine receptors in rat dorsal root ganglion neurons. *Neuroscience*. 2002; 115(1):1–5. [PubMed: 12401316]
- Liu JZ, Tozzi F, Waterworth DM, Pillai SG, Muglia P, Middleton L, Berrettini W, Knouff CW, Yuan X, Waeber G, Vollenweider P, Preisig M, Wareham NJ, Zhao JH, Loos RJ, Barroso I, Khaw KT, Grundy S, Barter P, Mahley R, Kesaniemi A, McPherson R, Vincent JB, Strauss J, Kennedy JL, Farmer A, McGuffin P, Day R, Matthews K, Bakke P, Gulsvik A, Lucae S, Ising M, Brueckl T, Horstmann S, Wichmann HE, Rawal R, Dahmen N, Lamina C, Polasek O, Zgaga L, Huffman J, Campbell S, Kooner J, Chambers JC, Burnett MS, Devaney JM, Pichard AD, Kent KM, Satler L, Lindsay JM, Waksman R, Epstein S, Wilson JF, Wild SH, Campbell H, Vitart V, Reilly MP, Li M, Qu L, Wilensky R, Matthai W, Hakonarson HH, Rader DJ, Franke A, Wittig M, Schafer A, Uda M, Terracciano A, Xiao X, Busonero F, Scheet P, Schlessinger D, St Clair D, Rujescu D, Abecasis GR, Grabe HJ, Teumer A, Volzke H, Petersmann A, John U, Rudan I, Hayward C, Wright AF, Kolcic I, Wright BJ, Thompson JR, Balmforth AJ, Hall AS, Samani NJ, Anderson CA, Ahmad T, Mathew CG, Parkes M, Satsangi J, Caulfield M, Munroe PB, Farrall M, Dominiczak A, Worthington J, Thomson W, Eyre S, Barton A, Mooser V, Francks C, Marchini J. Meta-analysis and imputation refines the association of 15q25 with smoking quantity. *Nat Genet*. 2010; 42(5):436–440. [PubMed: 20418889]
- Liu Q, Tang Z, Gan Y, Wu W, Kousari A, La Cava A, Shi FD. Genetic deficiency of beta2-containing nicotinic receptors attenuates brain injury in ischemic stroke. *Neuroscience*. 2014; 256:170–177. [PubMed: 24184117]
- Lombardo S, Maskos U. Role of the nicotinic acetylcholine receptor in Alzheimer's disease pathology and treatment. *Neuropharmacology*. 2015; 96(Pt B):255–262. [PubMed: 25514383]
- Lotfipour S, Byun JS, Leach P, Fowler CD, Murphy NP, Kenny PJ, Gould TJ, Boulter J. Targeted deletion of the mouse alpha2 nicotinic acetylcholine receptor subunit gene (Chrna2) potentiates nicotine-modulated behaviors. *J Neurosci*. 2013; 33(18):7728–7741. [PubMed: 23637165]
- Lou XY, Ma JZ, Payne TJ, Beuten J, Crew KM, Li MD. Gene-based analysis suggests association of the nicotinic acetylcholine receptor beta1 subunit (CHRNA1) and M1 muscarinic acetylcholine receptor (CHRM1) with vulnerability for nicotine dependence. *Hum Genet*. 2006; 120(3):381–389. [PubMed: 16874522]
- McKee SA, Harrison EL, O'Malley SS, Krishnan-Sarin S, Shi J, Tetrault JM, Picciotto MR, Petrakis IL, Estevez N, Balchunas E. Varenicline reduces alcohol self-administration in heavy-drinking smokers. *Biol Psychiatry*. 2009; 66(2):185–190. [PubMed: 19249750]

- McLaren W, Pritchard B, Rios D, Chen Y, Flicek P, Cunningham F. Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. *Bioinformatics*. 2010; 26(16): 2069–2070. [PubMed: 20562413]
- Meyer EL, Yoshikami D, McIntosh JM. The neuronal nicotinic acetylcholine receptors alpha 4* and alpha 6* differentially modulate dopamine release in mouse striatal slices. *J Neurochem*. 2008; 105(5):1761–1769. [PubMed: 18248619]
- Moretti M, Vailati S, Zoli M, Lippi G, Riganti L, Longhi R, Viegi A, Clementi F, Gotti C. Nicotinic acetylcholine receptor subtypes expression during rat retina development and their regulation by visual experience. *Molecular pharmacology*. 2004; 66(1):85–96. [PubMed: 15213299]
- Nakauchi S, Brennan RJ, Boulter J, Sumikawa K. Nicotine gates long-term potentiation in the hippocampal CA1 region via the activation of alpha2* nicotinic ACh receptors. *Eur J Neurosci*. 2007; 25(9):2666–2681. [PubMed: 17466021]
- Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic acids research*. 2003; 31(13):3812–3814. [PubMed: 12824425]
- Nuutinen S, Ahtee L, Tuominen RK. Time and brain region specific up-regulation of low affinity neuronal nicotinic receptors during chronic nicotine administration in mice. *Eur J Pharmacol*. 2005; 515(1–3):83–89. [PubMed: 15894304]
- Pakkanen JS, Jokitalo E, Tuominen RK. Up-regulation of beta2 and alpha7 subunit containing nicotinic acetylcholine receptors in mouse striatum at cellular level. *Eur J Neurosci*. 2005; 21(10): 2681–2691. [PubMed: 15926916]
- Parker SL, Fu Y, McAllen K, Luo J, McIntosh JM, Lindstrom JM, Sharp BM. Up-regulation of brain nicotinic acetylcholine receptors in the rat during long-term self-administration of nicotine: disproportionate increase of the alpha6 subunit. *Molecular pharmacology*. 2004; 65(3):611–622. [PubMed: 14978239]
- Pergadia ML, Agrawal A, Loukola A, Montgomery GW, Broms U, Saccone SF, Wang JC, Todorov AA, Heikkila K, Statham DJ, Henders AK, Campbell MJ, Rice JP, Todd RD, Heath AC, Goate AM, Peltonen L, Kaprio J, Martin NG, Madden PA. Genetic linkage findings for DSM-IV nicotine withdrawal in two populations. *Am J Med Genet B Neuropsychiatr Genet*. 2009; 150B(7):950–959. [PubMed: 19180564]
- Perkins KA, Lerman C, Mercincavage M, Fonte CA, Briski JL. Nicotinic acetylcholine receptor beta2 subunit (CHRNA2) gene and short-term ability to quit smoking in response to nicotine patch. *Cancer Epidemiol Biomarkers Prev*. 2009; 18(10):2608–2612. [PubMed: 19755656]
- Perry DC, Xiao Y, Nguyen HN, Musachio JL, Davila-Garcia MI, Kellar KJ. Measuring nicotinic receptors with characteristics of alpha4beta2, alpha3beta2 and alpha3beta4 subtypes in rat tissues by autoradiography. *J Neurochem*. 2002; 82(3):468–481. [PubMed: 12153472]
- Philibert RA, Todorov A, Andersen A, Hollenbeck N, Gunter T, Heath A, Madden P. Examination of the nicotine dependence (NICSNP) consortium findings in the Iowa adoption studies population. *Nicotine Tob Res*. 2009; 11(3):286–292. [PubMed: 19307444]
- Philip NS, Carpenter LL, Tyrka AR, Price LH. Nicotinic acetylcholine receptors and depression: a review of the preclinical and clinical literature. *Psychopharmacology (Berl)*. 2010; 212(1):1–12. [PubMed: 20614106]
- Picciotto MR, Zoli M, Rimondini R, Lena C, Marubio LM, Pich EM, Fuxe K, Changeux JP. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. *Nature*. 1998; 391(6663):173–177. [PubMed: 9428762]
- Pistillo F, Clementi F, Zoli M, Gotti C. Nicotinic, glutamatergic and dopaminergic synaptic transmission and plasticity in the mesocorticolimbic system: focus on nicotine effects. *Progress in neurobiology*. 2015; 124:1–27. [PubMed: 25447802]
- Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet*. 2006; 38(8): 904–909. [PubMed: 16862161]
- Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet*. 2007; 81(3):559–575. [PubMed: 17701901]

- Quick MW, Ceballos RM, Kasten M, McIntosh JM, Lester RA. Alpha3beta4 subunit-containing nicotinic receptors dominate function in rat medial habenula neurons. *Neuropharmacology*. 1999; 38(6):769–783. [PubMed: 10465681]
- Quik M, Polonskaya Y, Gillespie A, Jakowec M, Lloyd GK, Langston JW. Localization of nicotinic receptor subunit mRNAs in monkey brain by in situ hybridization. *J Comp Neurol*. 2000; 425(1): 58–69. [PubMed: 10940942]
- Rose JE. Multiple brain pathways and receptors underlying tobacco addiction. *Biochemical pharmacology*. 2007; 74(8):1263–1270. [PubMed: 17826746]
- Sabatelli M, Eusebi F, Al-Chalabi A, Conte A, Madia F, Luigetti M, Mancuso I, Limatola C, Trettel F, Sobrero F, Di Angelantonio S, Grassi F, Di Castro A, Moriconi C, Fucile S, Lattante S, Marangi G, Murdolo M, Orteschi D, Del Grande A, Tonali P, Neri G, Zollino M. Rare missense variants of neuronal nicotinic acetylcholine receptor altering receptor function are associated with sporadic amyotrophic lateral sclerosis. *Hum Mol Genet*. 2009; 18(20):3997–4006. [PubMed: 19628475]
- Saccone NL, Saccone SF, Hinrichs AL, Stitzel JA, Duan W, Pergadia ML, Agrawal A, Breslau N, Gruzka RA, Hatsukami D, Johnson EO, Madden PA, Swan GE, Wang JC, Goate AM, Rice JP, Bierut LJ. Multiple distinct risk loci for nicotine dependence identified by dense coverage of the complete family of nicotinic receptor subunit (CHRN) genes. *Am J Med Genet B Neuropsychiatr Genet*. 2009; 150B(4):453–466. [PubMed: 19259974]
- Saccone NL, Schwantes-An TH, Wang JC, Gruzka RA, Breslau N, Hatsukami D, Johnson EO, Rice JP, Goate AM, Bierut LJ. Multiple cholinergic nicotinic receptor genes affect nicotine dependence risk in African and European Americans. *Genes Brain Behav*. 2010; 9(7):741–750. [PubMed: 20584212]
- Saccone SF, Hinrichs AL, Saccone NL, Chase GA, Konvicka K, Madden PA, Breslau N, Johnson EO, Hatsukami D, Pomerleau O, Swan GE, Goate AM, Rutter J, Bertelsen S, Fox L, Fugman D, Martin NG, Montgomery GW, Wang JC, Ballinger DG, Rice JP, Bierut LJ. Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. *Hum Mol Genet*. 2007a; 16(1):36–49. [PubMed: 17135278]
- Saccone SF, Pergadia ML, Loukola A, Broms U, Montgomery GW, Wang JC, Agrawal A, Dick DM, Heath AC, Todorov AA, Maunu H, Heikkila K, Morley KI, Rice JP, Todd RD, Kaprio J, Peltonen L, Martin NG, Goate AM, Madden PA. Genetic linkage to chromosome 22q12 for a heavy-smoking quantitative trait in two independent samples. *Am J Hum Genet*. 2007b; 80(5):856–866. [PubMed: 17436240]
- Salas R, Pieri F, De Biasi M. Decreased signs of nicotine withdrawal in mice null for the beta4 nicotinic acetylcholine receptor subunit. *J Neurosci*. 2004; 24(45):10035–10039. [PubMed: 15537871]
- Salas R, Pieri F, Fung B, Dani JA, De Biasi M. Altered anxiety-related responses in mutant mice lacking the beta4 subunit of the nicotinic receptor. *J Neurosci*. 2003; 23(15):6255–6263. [PubMed: 12867510]
- Salminen O, Drapeau JA, McIntosh JM, Collins AC, Marks MJ, Grady SR. Pharmacology of alpha-conotoxin MII-sensitive subtypes of nicotinic acetylcholine receptors isolated by breeding of null mutant mice. *Molecular pharmacology*. 2007; 71(6):1563–1571. [PubMed: 17341654]
- Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, Grady SR. Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Molecular pharmacology*. 2004; 65(6):1526–1535. [PubMed: 15155845]
- Schlaepfer IR, Hoft NR, Collins AC, Corley RP, Hewitt JK, Hopfer CJ, Lessem JM, McQueen MB, Rhee SH, Ehringer MA. The CHRNA5/A3/B4 gene cluster variability as an important determinant of early alcohol and tobacco initiation in young adults. *Biol Psychiatry*. 2008; 63(11):1039–1046. [PubMed: 18163978]
- Sgard F, Charpentier E, Bertrand S, Walker N, Caput D, Graham D, Bertrand D, Besnard F. A novel human nicotinic receptor subunit, alpha10, that confers functionality to the alpha9-subunit. *Molecular pharmacology*. 2002; 61(1):150–159. [PubMed: 11752216]
- Stephens SH, Hartz SM, Hoft NR, Saccone NL, Corley RC, Hewitt JK, Hopfer CJ, Breslau N, Coon H, Chen X, Ducci F, Dueker N, Franceschini N, Frank J, Han Y, Hansel NN, Jiang C, Korhonen T, Lind PA, Liu J, Lyytikainen LP, Michel M, Shaffer JR, Short SE, Sun J, Teumer A, Thompson JR,

Vogelzangs N, Vink JM, Wenzlaff A, Wheeler W, Yang BZ, Aggen SH, Balmforth AJ, Baumeister SE, Beaty TH, Benjamin DJ, Bergen AW, Broms U, Cesarini D, Chatterjee N, Chen J, Cheng YC, Cichon S, Couper D, Cucca F, Dick D, Foroud T, Furberg H, Giegling I, Gillespie NA, Gu F, Hall AS, Hallfors J, Han S, Hartmann AM, Heikkila K, Hickie IB, Hottenga JJ, Jousilahti P, Kaakinen M, Kahonen M, Koellinger PD, Kittner S, Konte B, Landi MT, Laatikainen T, Leppert M, Levy SM, Mathias RA, McNeil DW, Medland SE, Montgomery GW, Murray T, Nauck M, North KE, Pare PD, Pergadia M, Ruczinski I, Salomaa V, Viikari J, Willemsen G, Barnes KC, Boerwinkle E, Boomsma DI, Caporaso N, Edenberg HJ, Francks C, Gelernter J, Grabe HJ, Hops H, Jarvelin MR, Johannesson M, Kendler KS, Lehtimäki T, Magnusson PK, Marazita ML, Marchini J, Mitchell BD, Nothen MM, Penninx BW, Raitakari O, Rietschel M, Rujescu D, Samani NJ, Schwartz AG, Shete S, Spitz M, Swan GE, Volzke H, Veijola J, Wei Q, Amos C, Cannon DS, Gruzza R, Hatsukami D, Heath A, Johnson EO, Kaprio J, Madden P, Martin NG, Stevens VL, Weiss RB, Kraft P, Bierut LJ, Ehringer MA. Distinct loci in the CHRNA5/CHRNA3/CHRNA4 gene cluster are associated with onset of regular smoking. *Genet Epidemiol.* 2013; 37(8):846–859. [PubMed: 24186853]

Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A.* 2003; 100(16):9440–9445. [PubMed: 12883005]

Subramaniam M, Dani JA. Dopaminergic and cholinergic learning mechanisms in nicotine addiction. *Annals of the New York Academy of Sciences.* 2015; 1349:46–63. [PubMed: 26301866]

Swan GE, Hops H, Wilhelmsen KC, Lessov-Schlaggar CN, Cheng LS, Hudmon KS, Amos CI, Feiler HS, Ring HZ, Andrews JA, Tildesley E, Benowitz N. A genome-wide screen for nicotine dependence susceptibility loci. *Am J Med Genet B Neuropsychiatr Genet.* 2006; 141B(4):354–360. [PubMed: 16671072]

Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, Whiteaker P, Marks MJ, Collins AC, Lester HA. Nicotine activation of $\alpha 4^*$ receptors: sufficient for reward, tolerance, and sensitization. *Science.* 2004; 306(5698):1029–1032. [PubMed: 15528443]

Thorgerirsson TE, Geller F, Sulem P, Rafnar T, Wiste A, Magnusson KP, Manolescu A, Thorleifsson G, Stefansson H, Ingason A, Stacey SN, Bergthorsson JT, Thorlacius S, Gudmundsson J, Jonsson T, Jakobsdottir M, Saemundsdottir J, Olafsdottir O, Gudmundsson LJ, Bjornsdottir G, Kristjansson K, Skuladottir H, Isaksson HJ, Gudbjartsson T, Jones GT, Mueller T, Gottsater A, Flex A, Aben KK, de Vegt F, Mulders PF, Isla D, Vidal MJ, Asin L, Saez B, Murillo L, Blondal T, Kolbeinsson H, Stefansson JG, Hansdottir I, Runarsdottir V, Pola R, Lindblad B, van Rij AM, Dieplinger B, Haltmayer M, Mayordomo JI, Kiemeny LA, Matthiasson SE, Oskarsson H, Tyrfinngsson T, Gudbjartsson DF, Gulcher JR, Jonsson S, Thorsteinsdottir U, Kong A, Stefansson K. A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature.* 2008; 452(7187):638–642. [PubMed: 18385739]

Thorgerirsson TE, Gudbjartsson DF, Surakka I, Vink JM, Amin N, Geller F, Sulem P, Rafnar T, Esko T, Walter S, Gieger C, Rawal R, Mangino M, Prokopenko I, Magi R, Keskitalo K, Gudjonsson IH, Gretarsdottir S, Stefansson H, Thompson JR, Aulchenko YS, Nelis M, Aben KK, den Heijer M, Dirksen A, Ashraf H, Soranzo N, Valdes AM, Steves C, Uitterlinden AG, Hofman A, Tonjes A, Kovacs P, Hottenga JJ, Willemsen G, Vogelzangs N, Doring A, Dahmen N, Nitz B, Pergadia ML, Saez B, De Diego V, Lezcano V, Garcia-Prats MD, Ripatti S, Perola M, Kettunen J, Hartikainen AL, Pouta A, Laitinen J, Isohanni M, Huei-Yi S, Allen M, Krestyaninova M, Hall AS, Jones GT, van Rij AM, Mueller T, Dieplinger B, Haltmayer M, Jonsson S, Matthiasson SE, Oskarsson H, Tyrfinngsson T, Kiemeny LA, Mayordomo JI, Lindholt JS, Pedersen JH, Franklin WA, Wolf H, Montgomery GW, Heath AC, Martin NG, Madden PA, Giegling I, Rujescu D, Jarvelin MR, Salomaa V, Stumvoll M, Spector TD, Wichmann HE, Metspalu A, Samani NJ, Penninx BW, Oostra BA, Boomsma DI, Tiemeier H, van Duijn CM, Kaprio J, Gulcher JR, McCarthy MI, Peltonen L, Thorsteinsdottir U, Stefansson K. Sequence variants at CHRNA3-CHRNA4 and CYP2A6 affect smoking behavior. *Nat Genet.* 2010; 42(5):448–453. [PubMed: 20418888]

Tobacco and Genetics Consortium. Genome-wide meta-analyses identify multiple loci associated with smoking behavior. *Nat Genet.* 2010; 42(5):441–447. [PubMed: 20418890]

Turner JR, Kellar KJ. Nicotinic cholinergic receptors in the rat cerebellum: multiple heteromeric subtypes. *J Neurosci.* 2005; 25(40):9258–9265. [PubMed: 16207885]

Wang JC, Cruchaga C, Saccone NL, Bertelsen S, Liu P, Budde JP, Duan W, Fox L, Gruzza RA, Kern J, Mayo K, Reyes O, Rice J, Saccone SF, Spiegel N, Steinbach JH, Stitzel JA, Anderson MW, You

- M, Stevens VL, Bierut LJ, Goate AM. Risk for nicotine dependence and lung cancer is conferred by mRNA expression levels and amino acid change in CHRNA5. *Hum Mol Genet.* 2009a; 18(16):3125–3135. [PubMed: 19443489]
- Wang JC, Grucza R, Cruchaga C, Hinrichs AL, Bertelsen S, Budde JP, Fox L, Goldstein E, Reyes O, Saccone N, Saccone S, Xuei X, Bucholz K, Kuperman S, Nurnberger J Jr, Rice JP, Schuckit M, Tischfield J, Hesselbrock V, Porjesz B, Edenberg HJ, Bierut LJ, Goate AM. Genetic variation in the CHRNA5 gene affects mRNA levels and is associated with risk for alcohol dependence. *Mol Psychiatry.* 2009b; 14(5):501–510. [PubMed: 18414406]
- Wang S, DvdV A, Xu Q, Seneviratne C, Pomerleau OF, Pomerleau CS, Payne TJ, Ma JZ, Li MD. Significant associations of CHRNA2 and CHRNA6 with nicotine dependence in European American and African American populations. *Hum Genet.* 2014; 133(5):575–586. [PubMed: 24253422]
- Wei J, Chu C, Wang Y, Yang Y, Wang Q, Li T, Zhang L, Ma X. Association study of 45 candidate genes in nicotine dependence in Han Chinese. *Addict Behav.* 2012; 37(5):622–626. [PubMed: 22309839]
- Wilens TE, Decker MW. Neuronal nicotinic receptor agonists for the treatment of attention-deficit/hyperactivity disorder: focus on cognition. *Biochemical pharmacology.* 2007; 74(8):1212–1223. [PubMed: 17689498]
- Won WY, Park B, Choi SW, Kim L, Kwon M, Kim JH, Lee CU, Shin HD, Kim DJ. Genetic Association of CHRNA3 and CHRNA6 Gene Polymorphisms with Nicotine Dependence Syndrome Scale in Korean Population. *Psychiatry Investig.* 2014; 11(3):307–312.
- Xie P, Kranzler HR, Krauthammer M, Cosgrove KP, Oslin D, Anton RF, Farrer LA, Picciotto MR, Krystal JH, Zhao H, Gelernter J. Rare nonsynonymous variants in alpha-4 nicotinic acetylcholine receptor gene protect against nicotine dependence. *Biol Psychiatry.* 2011; 70(6):528–536. [PubMed: 21683344]
- Xu Z, Taylor JA. SNPinfo: integrating GWAS and candidate gene information into functional SNP selection for genetic association studies. *Nucleic acids research.* 2009; 37:W600–605. [PubMed: 19417063]
- Yang J, Wang S, Yang Z, Hodgkinson CA, Iarikova P, Ma JZ, Payne TJ, Goldman D, Li MD. The contribution of rare and common variants in 30 genes to risk nicotine dependence. *Mol Psychiatry.* 2014
- Zoli M, Moretti M, Zanardi A, McIntosh JM, Clementi F, Gotti C. Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. *J Neurosci.* 2002; 22(20):8785–8789. [PubMed: 12388584]

Table 1Associations between individual rare *CHRN* variants and different neuropsychiatric disorders

Human Diseases	Ethnicity	Dataset name	SNP # (total)	SNP # (p<0.05)	Most significant		Minor allele frequency (N)	
					SNP	Gene	Minimal p value	Affected Unaffected
Nicotine dependence	EA	COGEND+UW-TTURC	268	8	rs60757049	<i>CHRNA4</i>	0.0104	0.004 (836) 0.047 (80)
Nicotine dependence	AA	COGEND+UW-TTURC	235	4	rs2231540	<i>CHRNA10</i>	0.0021	0.124 (392) 0.044 (79)
Alcoholism	EA	SAGE+COGA	429	15	rs1346725	<i>CHRNA2</i>	0.0082	0.001 (1409) 0.005 (1518)
AD+ND	EA	SAGE+COGA	413	6	rs13040031	<i>CHRNA4</i>	0.0175	0.032 (1409) 0.016 (1518)
Alcoholism	AA	SAGE+COGA	620	15	rs76706995	<i>CHRNA9</i>	5.4×10^{-4}	0.031 (681) 0.010 (508)
Schizophrenia	EA	GAIN	203	4	rs2231547	<i>CHRNA10</i>	0.0244	0.031 (1351) 0.044 (1378)
Schizophrenia	AA	GAIN	596	10	rs79576636	<i>CHRNA7</i>	8.4×10^{-4}	0.008 (1195) 0.0006 (954)
Schizophrenia	EA	MGS_nonGAIN	212	7	rs1399190	<i>CHRNA7</i>	0.0182	0.006 (1437) 0.002 (1347)
Major Depression	CA	PRSC	412	5	rs6011783	<i>CHRNA4</i>	0.0221	0.036 (1805) 0.026 (1820)
Alzheimer's disease	EA	GenADA	226	4	rs8834	<i>CHRNA7</i>	0.0018	0.054 (806) 0.022 (782)
Parkinson's Disease	CA	NGRC	398	26	rs62518191	<i>CHRNA3</i>	8.5×10^{-4}	0.024 (2000) 0.014 (1986)
Parkinson's Disease	CA	Ing_corieLL_pd	388	8	rs75628551	<i>CHRNA3</i>	9.8×10^{-4}	0.0006 (940) 0.009 (801)
ALS	CA	GRU	297	9	rs66850163	<i>CHRNA7</i>	0.0076	0.041 (261) 0.007 (246)
Ischemic Stroke	CA	ISGS	302	23	rs16956147	<i>CHRNA7</i>	0.0075	0.014 (219) 0.045 (266)
Early Onset Stroke	EA	GEOS × 3	321	3	rs2273504	<i>CHRNA4</i>	0.0040	0.074 (372) 0.028 (430)
Early Onset Stroke	AA	GEOS × 3	538	8	rs28454587	<i>CHRNA9</i>	0.0253	0.041 (309) 0.018 (290)

Dataset names refer to dbGaP. Only the most significant risk markers with minimal p values are listed; "minimal p-value" is the smallest p-value among all the SNPs in all genes studied for a particular dataset. ALS, Amyotrophic Lateral Sclerosis; AA, African-American; EA, European-American; CA, Caucasian; N, sample size of subjects. Dataset names refer to dbGaP and references [GenADA: Li et al. Arch Neurol. 2008;65(1):45–53; Filippini et al. Neuroimage. 2009;44(3):724–728].

Table 2

p values for associations between global *CHRN* rare variant constellation and diseases

Diseases	Dataset	T1	T5	Fp
Nicotine dependence	COGEND+UW-TTURC (EA)	5.0×10^{-18}	8.4×10^{-54}	1.5×10^{-58}
Nicotine dependence	COGEND+UW-TTURC (AA)	1.3×10^{-4}	1.9×10^{-35}	3.9×10^{-33}
Alcoholism	SAGE+COGA (EA)	0.544	0.213	0.267
AD+ND	SAGE+COGA (EA)	1.5×10^{-8}	8.6×10^{-17}	1.0×10^{-17}
Alcoholism	SAGE+COGA (AA)	7.9×10^{-4}	3.5×10^{-6}	1.7×10^{-6}
AD+ND	SAGE+COGA (AA)	0.987	0.569	0.626
Schizophrenia	GAIN (EA)	0.665	0.523	0.435
Schizophrenia	GAIN (AA)	0.302	0.033	0.024
Schizophrenia	MGS_nonGAIN (EA)	0.996	0.295	0.274
Major Depression	PRSC	0.127	0.198	0.197
Alzheimer's disease	GenADA	0.711	0.539	0.400
Parkinson's Disease	NGRC	0.669	0.197	0.206
Parkinson's Disease	Ing_corieII_pd	0.177	7.1×10^{-3}	8.0×10^{-3}
ALS	GRU	0.523	0.149	0.152
Ischemic Stroke	ISGS	0.427	0.324	0.405
Early Onset Stroke	GEOS $\times$ 3	0.311	0.054	0.061
Early Onset Stroke	GEOS $\times$ 3	0.371	0.477	0.390

EA, European-American; AA, African-American. Dataset names correspond to Table 1. T1, T5 and Fp, association tests using SCORE-Seq: (1) In the T1 and T5 tests, the MAF upper bound thresholds were fixed at 0.01 and 0.05, respectively, and the weight was fixed at 1; (2) In the Fp tests, the MAF upper bound threshold was fixed at 0.05, but the weight was $1/\sqrt{p(1-p)}$ where p was the estimated MAF with pseudo counts in the pooled sample. $\alpha=0.05/15=3.3 \times 10^{-3}$ for T1 test.

Table 3

p values for associations between rare *CHRN* variant constellation at each gene/region and diseases

Gene/region	Chr	Length (bp)	Number of rare SNPs	Nicotine dependence in 1 st EAs			Nicotine dependence in 1 st AAs			ND+AD in 2 nd EAs			Alcohol dependence in 2 nd AAs		
				T1	T5	Fp	T1	T5	Fp	T1	T5	Fp	T1	T5	Fp
CHRN ^{B2}	1	12098	38	N/A	1.3×10 ⁻¹⁸	4.9×10 ⁻¹⁸	N/A	2.4×10 ⁻⁶	9.5×10 ⁻¹²	0.580	5.1×10 ⁻⁵	3.0×10 ⁻³	0.132	1.9×10 ⁻³	1.2×10 ⁻³
CHRNA1	2	16881	112				0.030	2.5×10 ⁻¹¹	1.2×10 ⁻¹⁴						
CHRN ^D -CHRN ^G	2	20169	82							5.9×10 ⁻⁵	1.5×10 ⁻⁷	9.4×10 ⁻⁸			
CHRNA9	4	19889	167	0.034	5.0×10 ⁻¹⁹	6.1×10 ⁻³⁰				0.020	6.5×10 ⁻⁸	1.9×10 ⁻⁸	0.039	4.0×10 ⁻⁶	3.5×10 ⁻⁶
CHRNA2	8	19536	120	2.1×10 ⁻⁴	5.2×10 ⁻¹³	3.3×10 ⁻¹²									
CHRN ^{B3} -CHRNA6	8	71368	329	1.3×10 ⁻⁴	1.5×10 ⁻¹⁵	3.8×10 ⁻¹⁶									
CHRNA10	11	7622	56	4.3×10 ⁻⁶	3.4×10 ⁻²⁹	1.2×10 ⁻²⁵							7.5×10 ⁻³	9.1×10 ⁻⁶	1.4×10 ⁻⁶
CHRNA7	15	36080	565	1.1×10 ⁻⁶	1.5×10 ⁻¹⁹	7.6×10 ⁻²²	4.9×10 ⁻³	2.4×10 ⁻²⁵	6.1×10 ⁻²⁷	6.5×10 ⁻³	8.9×10 ⁻⁹	3.5×10 ⁻⁹	4.5×10 ⁻³	5.1×10 ⁻⁴	4.0×10 ⁻⁴
CHRNA5-CHRNA3-CHRN ^{B4}	15	90067	374	2.2×10 ⁻⁴	1.9×10 ⁻²⁵	4.6×10 ⁻³²	2.6×10 ⁻³	1.5×10 ⁻¹⁶	1.3×10 ⁻²²	8.0×10 ⁻⁴	1.1×10 ⁻⁹	9.5×10 ⁻¹¹			
CHRN ^E	17	5360	35	5.3×10 ⁻⁴	4.9×10 ⁻¹⁵	9.1×10 ⁻¹⁴									
CHRNA4	20	18087	175	8.3×10 ⁻³	9.8×10 ⁻³⁹	1.9×10 ⁻³⁹				9.9×10 ⁻³	2.7×10 ⁻¹²	2.3×10 ⁻¹⁰			

T1, T5 and Fp, association tests using SCORE-Seq. EA, European-American; AA, African-American; ND+AD, nicotine and alcohol co-dependence. N/A, not available because of lack of rare variants with MAF<0.01. α=0.05/12=0.004 in which “12” is the number of gene regions. P values larger than α are not listed in the table.

Table 4

Bioinformatics analysis for the missense rare variants

SNP	Gene	AAs	Bioinformatics analysis
rs61737716	CHRNA1	E/D	probably damaging
rs2289080	CHRNA1	A/T	splicing (ESE or ESS)
rs35327613	CHRNA3	K/E	
rs2231546	CHRNA10	G/S	
rs2231547	CHRNA10	E/A	deleterious; conservative (1.000)
rs55719530	CHRNA10	T/N	deleterious; probably damaging; splicing (ESE or ESS)
rs16969968	CHRNA5	D/N	
rs79109919	CHRNA5	L/Q	deleterious; probably damaging
rs79835149	CHRNA5	Y/*	stop gained
rs12914008	CHRNA4	T/I	deleterious; possibly damaging; splicing (ESE or ESS); conservative (0.941)
rs56095004	CHRNA4	R/Q	splicing (ESE or ESS)
rs61737499	CHRNA4	T/I	
rs76927517	CHRNA1	I/T	deleterious; possibly damaging; 538bp-CpG
rs4790235	CHRNA	G/V	probably damaging; splicing (ESE or ESS)

AAs, amino acid changes; splicing (ESE or ESS), these SNPs are located in exonic splicing silencers or enhancers; deleterious/probably damaging, these SNPs are predicted by SIFT/Polyphen to significantly affect protein function or structure; conservative (score), these SNPs are predicted to be conservative across species; CpG, this SNP is located within a 538bp methylated CpG island;

* stop gained, this SNP results in a premature stop codon, leading to a shortened transcript.

Table 5

Chrna gene expression at whole brain and different brain areas of BXD mice

Gene	Location (Chr, Mb)	Whole brain	Cortex	Striatum	NAc	Hypothalamus	Hippocampus	Amygdala	Mid-brain	VTA	Cerebellum	Pituitary
Chrnb2	Chr3: 89.561004	8.39	9.54	8.65	7.93	11.15	10.22	10.66	9.98	7.90	7.94	10.60
Chrna1	Chr2: 73.403930	7.10	7.10		7.01	7.08	7.90				7.54	7.90
Chrmd	Chr1: 89.095164	9.15			8.58		7.54			8.54	7.70	
Chrng	Chr1: 89.107206	7.55		7.29		7.49	8.62	7.43			8.00	7.44
Chrna9	Chr5: 66.367985	7.35	7.30			7.03	7.50	7.34	7.90		7.08	7.55
Chrna2	Chr14: 66.771218	7.43	7.14	7.33			7.85	7.59	8.87			
Chrnb3	Chr8: 28.504645	7.85	7.11			7.11	7.25			7.65		9.03
Chrna6	Chr8: 28.513939	8.73			7.79	7.05	7.13		10.37	8.97	7.23	8.68
Chrna10	Chr7: 109.259780		7.08				7.43	7.44				
Chrna7	Chr7: 70.243861	7.98	7.19	9.33	7.49	9.78	10.88	9.95	8.28	7.53	7.16	7.50
Chrna5	Chr9: 54.852890		7.22				8.44	7.45				
Chrna3	Chr9: 54.860390	9.35			7.22	8.20	8.32			7.60	7.61	
Chrnb4	Chr9: 54.877893	7.23		7.58		8.26	8.65	8.18	8.23			8.17
Chrnb1	Chr11: 69.597667			7.01			7.50					8.11
Chme	Chr11: 70.428505	8.13	7.05		7.12	7.32	7.63	7.34				7.20
Chrna4	Chr2: 180.759407	9.48	10.05	9.02	8.29	11.09	9.95	10.73	9.64	8.71	8.30	9.23

The order of gene list corresponds to Table 3. Only the expression values > 7 were listed.