Contribution of Variants in *CHRNA5/A3/B4* Gene Cluster on Chromosome 15 to Tobacco Smoking: From Genetic Association to Mechanism

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Abstract Cigarette smoking is the major cause of preventable death and morbidity throughout the world. Many compounds are present in tobacco, but nicotine is the primary addictive one. Nicotine exerts its physiological and pharmacological roles in the brain through neuronal nicotinic acetylcholine receptors (nAChRs), which are ligand-gated ion channels consisting of five membrane-spanning subunits that can modulate the release of neurotransmitters, such as dopamine, glutamate, and GABA and mediate fast signal transmission at synapses. Considering that there are 12 nAChR subunits, it is highly likely that subunits other than α4 and β2, which have been intensively investigated, also are involved in nicotine addiction. Consistent with this hypothesis, a number of genome-wide association studies (GWAS) and subsequent candidate gene-based associated studies investigating the genetic variants associated with nicotine dependence (ND) and smoking-related phenotypes have shed light on the *CHRNA5/A3/B4* gene cluster on chromosome 15, which encodes the α5, α3, and β4 nAChR subunits, respectively. These studies demonstrate two groups of risk variants in this region. The first one is marked by single nucleotide polymorphism (SNP) rs16969968 in exon 5 of *CHRNA5*, which changes an aspartic acid residue into asparagine at position 398 (D398N) of the α5 subunit protein sequence, and it is tightly linked SNP rs1051730 in *CHRNA3*. The second one is SNP rs578776 in the 3′-untranslated region (UTR) of *CHRNA3*, which has a low correlation with rs16969968. Although the detailed molecular mechanisms underlying these associations remain to be further elucidated, recent findings have shown that α5* (where "*" indicates the presence of additional subunits) nAChRs located in the medial habenulo-interpeduncular nucleus (mHb-IPN) are involved in the control of nicotine self-administration in rodents. Disruption of α5* nAChR signaling diminishes the aversive effects of nicotine on the mHb-IPN pathway and thereby permits more nicotine consumption. To gain a better understanding of the function of the highly significant genetic variants identified in this region in controlling smoking-related behaviors, in this communication, we provide an up-to-date review of the progress of studies focusing on the *CHRNA5/A3/B4* gene cluster and its role in ND.

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Introduction

Cigarette smoking is one of the most significant public health problems in both developed and developing countries. Although new efficacious techniques for smoking cessation have helped to reduce the number of smokers significantly, there were still approximately 38 million tobacco users in the USA and 1 billion worldwide in 2012 [1]. Of these smokers, approximately 60 % are nicotine dependent [2]. The burden of smoking-related diseases and the negative economic impact on society caused by cigarette smoking is staggering. According to the World Health Organization's report, approximately five million people each year die of smoking-related illnesses [3], making smoking the largest cause of preventable death in the world, and if the current trend continues, the worldwide death toll caused by tobacco smoking will rise to eight million annually by 2030 [4]. Moreover, smoking has

various detrimental effects on physical health that often are serious, carrying significant risks of cardiovascular diseases, respiratory diseases, and lung cancer, among other ailments.

There are approximately 4000 compounds in cigarette smoke; however, nicotine is the primary component responsible for the development of nicotine dependence (ND) [5]. Nicotine exerts its pharmacological and physiological roles in the brain through neuronal nicotinic acetylcholine receptors (nAChRs), which are widely distributed in the central and peripheral nervous systems. The nAChRs are ligand-gated ion channels consisting of five membrane-spanning subunits [6] that can modulate the release of neurotransmitters such as dopamine (DA), GABA, and glutamate [7] and mediate fast signal transmission at synapses [8]. There are 12 neuronal acetylcholine receptor subunits, with nine α subunits ($\alpha2$–$\alpha10$) and three β subunits ($\beta2$–$\beta4$) [6, 9, 10]. These subunits arrange in numerous distinct pentameric nAChRs, resulting in receptors that differ in distribution throughout the body and in biologic functions and other pharmacologic properties [11]. Binding of nicotine to nAChRs forms the molecular basis for the reward of nicotine and, eventually, the development of ND. Thus, nAChRs represent not only plausible candidate risk factors for ND but also targets for drugs for treating ND and other psychiatric disorders.

Abundant data from twin studies demonstrate that along with environmental factors, genetic variations are responsible for ND, with an estimated heritability of about 50 % [12–16]. To identify susceptibility loci and genetic variants for ND and its related phenotypes, many studies have been conducted using various approaches such as genome-wide linkage analysis, candidate gene-based association, and genome-wide association studies (GWAS). Of the genetic variants found to be associated with ND, the variants in the *CHRNA5/A3/B4* gene cluster on chromosome 15, which encodes the $\alpha5$, $\alpha3$, and $\beta4$ subunits [17–20], have received much attention in the past several years. Importantly, the variants in this gene cluster have been associated, not only with ND, but with lung cancer [21–23]. As a result of this genetic research, new effort has been expended to understand how variants in this region impact ND and its related phenotypes at the molecular level.

Replication of genetic association between the variants in the *CHRNA5/A3/B4* gene cluster and ND increases the validity of these findings. At the same time, it stimulates interest in exploring the molecular mechanisms of variants within this gene cluster underlying ND. Of the significant variants in this gene cluster, single nucleotide polymorphism (SNP) rs16969968 appears to be the most attractive as an ND factor, as it results in an amino acid change from aspartate to asparagine at position 398 of the nicotinic receptor $\alpha5$ subunit protein sequence. How the clustered nAChR subunits function in the development of ND is still unclear, although evidence from mouse models with knockout (KO) or mutations of nAChR subunits, especially the $\alpha5$ subunit, suggests that disruption of $\alpha5*$ nAChR signaling diminishes the stimulatory effects of nicotine on the medial habenulo-interpeduncular nucleus (mHb-IPN) pathway and thereby permits consumption of greater quantities of nicotine [24]. Hence, it was thought that variants in the *CHRNA5/A3/B4* gene cluster play an important role in ND through the aversive effect of nicotine on the mHb-IPN pathway, whereas there are few reports concerning the reinforcing effect of nicotine in ventral tegmental area (VTA) DA neurons [25].

To gain a better understanding of the genetic factors that contribute to ND and other smoking-related phenotypes, in this review, we first focus on the significant association between the variants detected in the *CHRNA5/A3/B4* gene cluster and smoking-related phenotypes, and then present mechanisms that could explain such associations at the molecular level.

Association Between Common Variants in the *CHRNA5/A3/B4* Gene Cluster and Smoking-Related Phenotypes

Nicotine Dependence

ND, as well as addiction to any other substance, is a complicated phenotype. It involves many symptoms, consisting of early-morning smoking, heavier smoking, tolerance, and ease of relapse after quitting. More importantly, the development of ND is not a sudden event; it has to go through a transition from experimental smoking with the first puff to regular smoking and finally to the establishment of ND [26]. There are a series of assessment tools for ND; the more commonly used ones are the Fagerström test for nicotine dependence (FTND) [27] and the *Diagnostic and Statistical Manual for Mental Disorders (4th edition)* (DSM-IV) [28]. Although both scales are commonly used to evaluate the severity of ND, there exists a limited correlation between the two tools [27], because each focuses on different aspects of ND. The FTND is a simplified measure compared with the DSM-IV, which lays particular emphasis on the number of cigarettes smoked per day (CPD) and the time from waking to the first cigarette, whereas DSM-IV mainly emphasizes the behavioral and emotional aspects of addiction. Thus, when one considers the definition of ND using the FTND, we usually choose CPD to represent it, because of its easy measurement and appropriate matching to ND.

The first report concerning the contribution of variants in the *CHRNA5/A3/B4* gene cluster to ND was published by Saccone et al. in 2007 [17]. In this study, the authors examined 879 light smokers who had no symptoms of dependence, with an FTND score of 0, and 1050 heavy smokers, with an FTND score of >4.0, focusing on the transition from regular smoking to addiction. Among 3713 SNPs in more than 300 candidate genes analyzed, multiple risk SNPs were found in the *CHRN*

A5/A3/B4 gene cluster, with the most compelling evidence for a risk allele coming from a non-synonymous SNP rs16969968 in the α5 nicotine receptor subunit gene (*CHRNA5*) (p=6.4× 10^{-4}). Furthermore, this SNP exhibited a recessive mode of inheritance, resulting in individuals with one copy of the risk allele A having a 1.1-fold increase in the risk of developing ND once exposed to cigarette smoking, whereas there was a 2-fold increase with the AA genotype compared with subjects having no copy. Since then, numerous candidate gene-based analyses and large-scale GWAS, together with several meta-analyses [29–31] which elaborated on Vandenbergh's literature [32] have focused on the association of polymorphisms in the *CHRNA5/A3/B4* gene cluster with ND across different populations, leading to the conclusion that variants in this gene cluster contribute to the development of heavy smoking and ND [17–22, 26]. Together, these studies demonstrate two groups of risk variants in the cluster. The first one is marked by SNP rs16969968 in exon 5 of *CHRNA5*, which changes an aspartic acid residue into asparagine at position 398 (D398N) of the α5 subunit protein sequence, or its tightly linked SNP rs1051730 in *CHRNA3*. The other is SNP rs578776 in the 3′-untranslated region (UTR) of *CHRNA3*, which has a low linkage disequilibrium (LD) with rs16969968 (Table 1).

The association of these SNPs with ND can be modified by different factors. For instance, Weiss et al. [19] reported that individuals who became regular smokers before the age of 16 showed a signification association between SNP rs16969968 and the severity of ND, whereas Grucza et al. [38] found that the same SNP exhibited its effects mainly on late-onset smokers, after 16 years of age. What causes such inconsistent results remains to be investigated. In addition, other environmental factors, such as parental monitoring [39], childhood adversity [40], and peer smoking [41] have been reported to influence the association between SNPs rs16969968 or rs1051730 and ND.

On the other hand, there are a few reports concerning the effect of common variants in *CHRNB4* on ND. Three independent GWAS meta-analyses revealed the importance of the *CHRNA5/A3/B4* gene cluster in influencing ND, but failed to identify any SNP in the β4 receptor subunit gene as a contributor to the genetic association signal for heavy smoking [29, 42, 43]. Thus, for the time being, we are not clear on whether common variants in *CHRNB4* play any role in the development of ND, although such a role is theoretically possible because of the high LD patterns across *CHRNA5*, *CHRNA3*, and *CHRNB4* (Fig. 1).

Lung Cancer

Lung cancer, which can be divided into two major histopathologic types (small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC)), is the leading cause of cancer-related deaths throughout the world [44]. Among multiple risk factors associated with lung cancer, cigarette smoking is the most important one, as many carcinogens are present in cigarette smoke and others, such as (4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone) (NNK) and (*N*-nitrosonornicotine) (NNN), are metabolized from nicotine [45, 46]. Both of these compounds can stimulate the growth [47] or inhibit apoptosis [48] of lung cancer cells.

In parallel with the studies of ND, several SNPs within the *CHRNA5/A3/B4* gene cluster seem to increase the risk of lung cancer according to several GWAS and candidate gene-based association studies [21, 22, 49, 50]. Hung et al. [22] first found that SNP rs16969968 was robustly associated with lung cancer after studying nearly 317,139 SNPs in 4614 subjects of European descent. Since then, this finding has been replicated in different ethnic populations [49, 51–53]. Furthermore, Saccone et al. [29] demonstrated the presence of a significant association between rs16969968 and lung cancer (p<10^{-20}) in a meta-analysis of six datasets of European-ancestry subjects (N=13,614) [29]. However, whether the association of this SNP with lung cancer is directly or indirectly mediated by the variant's association with ND has been the subject of extensive debate in the past several years. One group of investigators favoring a direct role of variants in the *CHRNA5/A3/B4* gene cluster in lung cancer reasoned that the association was observed even in non-smokers [22] and remained significant after adjustment for smoking quantity [54, 55]. The other group, preferring an indirect role of the variant in lung cancer, argued that the studies failed to detect a significant association between the variant and lung cancer in never smokers [56]. The inaccurate measurement of uptake of carcinogens by self-reported CPD supports this view [57].

There might have some other elements, such as different ethnic backgrounds of the populations examined, sample sizes, and measurement strategies for smoking-related phenotypes, which contribute to the above-mentioned conflict. For example, the populations used in most of these studies were of European origin [21, 22], a group that has a 37–43 % frequency of the rs16969968 A allele, whereas the A nucleotide is not detected or is uncommon in African, East Asian, and Native American populations [18]. Consequently, the association between variants in the *CHRNA5/A3/B4* gene cluster and lung cancer needs to be further investigated in well-designed studies, especially in other ethnic samples.

Smoking Initiation and Cessation

Cigarette smoking can be divided into three behaviors: initiation, ND, and cessation. Many variables influence the three processes, including age, education, social status, and so on. Although the variants in the *CHRNA5/A3/B4* gene cluster are strongly associated with ND and smoking quantity, this region appears to play a smaller or less significant role in smoking initiation and cessation.

Table 1 Reported association of rs16969968, rs1051730, and rs578776 with nicotine dependence

dbSNP ID	Sample origin	Sample size	Cases (N)	Controls (N)	Minor allele frequency	Odds ratio	p value	Reference
rs16969968 (*CHRNA5*)	European (USA+Australia)	1929	1050	879	0.38	–[a]	6.42E−04	[17]
	European (USA+Australia)	1929	1050	879	0.383	1.31	1.30E−04	[33]
	Caucasian	1236	955	281	0.34	–[a]	7.00E−03	[18]
	European American	1968	1093	875	0.378	1.37	6.30E−08	[20]
	European American	377	271	106	0.415	1.79	9.00E−04	[19]
	European American	2062	1063	999	0.35	1.4	4.14E−07	[34]
	European (meta)	24,807	14,452	10,355	–[a]	1.327	5.96E−31	[30]
	German (3 cohorts)	5561	–[a]	–[a]	0.38	1.18	1.90E−04	[35]
	Mixed ethnic ancestry	571	–[a]	–[a]	0.357	–[a]	<0.0001	[36]
	Caucasian	3441	–[a]	–[a]	0.41	–[a]	1.10E−04	[37]
rs1051730 (*CHRNA3*)	USA+Australia	1929	1050	879	0.38	–[a]	9.93E−04	[17]
	European (USA+Australia)	1929	1050	879	0.382	1.3	2.01E−04	[33]
	Caucasian	1236	955	281	0.32	–[a]	2.00E−02	[18]
	European American	1933	1073	860	0.378	1.37	9.30E−08	[20]
	European American	377	271	106	0.415	1.79	9.00E−04	[19]
	European American	2062	1063	999	0.349	1.4	5.88E−07	[34]
	German (3 cohorts)	5561	–[a]	–[a]	0.38	1.19	7.50E−05	[35]
	Mixed ethnic ancestry	571	–[a]	–[a]	0.358	–[a]	<0.0001	[36]
	Caucasian	3441	–[a]	–[a]	0.41	–[a]	1.50E−04	[37]
rs578776 (*CHRNA3*)	USA+Australia	1929	1050	879	0.22	–[a]	3.08E−04	[17]
	European (USA+Australia)	1929	1050	879	0.241	0.746	1.06E−04	[33]
	Caucasian	1236	955	281	0.28	–[a]	9.00E−03	[18]
	European American	1564	707	839	0.244	0.75	1.37E−06	[20]
	European American	377	271	106	0.218	0.6	4.80E−03	[19]
	European (meta)	22,915	13,391	9524	–[a]	0.776	1.38E−25	[30]
	German (3 cohorts)	5561	–[a]	–[a]	0.26	0.85	9.30E−04	[35]
	Mixed ethnic ancestry	571	–[a]	–[a]	0.331	–[a]	2.40E−03	[36]
	Caucasian	3441	–[a]	–[a]	0.24	–[a]	2.00E−02	[37]

[a] Not available from original report

Thorgeirsson et al. [50] reported that the variants in *CHRN A5/A3/B4* did not influence smoking initiation and experimentation. Similarly, Lips et al. [58] and Kaur-Knudsen et al. [54] also concluded that the variants in the cluster on chromosome 15 did not play a role in identifying non-smokers and smokers. At the same time, Maes et al. [59] showed that the SNPs associated with ND did not show a significant association with either smoking initiation or regular smoking in a twin study. On the other hand, Sherva et al. [60] reported an association between rs16969968 in the *CHRNA5* gene and enhanced pleasurable responses to initial cigarette smoking, suggesting that phenotypes related to subjective experiences during smoking experimentation may mediate the development of ND. Meanwhile, Stephens et al. [61] conducted a meta-analysis
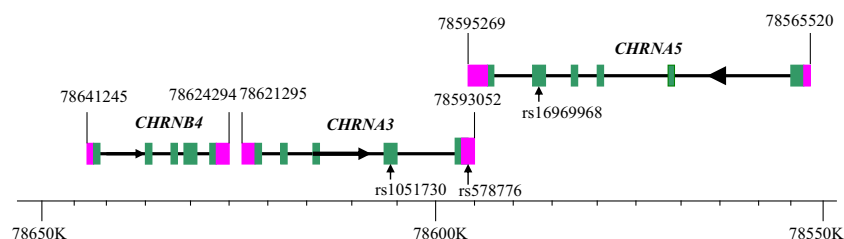

Fig. 1 Schematic diagram of the human *CHRNA5/A3/B4* cluster. *Horizontal black arrows* indicate the direction of transcription of each gene. *Green* and *pink rectangles* indicate exons and untranslated regions, respectively, while *horizontal black lines* represent introns. The genetic variants (rs1051730, rs578776, and rs16969968) significantly associated with ND are indicated by *vertical arrows*

including 56,034 subjects in 41 studies spanning nine countries, which showed a significant association of rs578776 with age of first regular tobacco use ($\beta=0.02$; $p=0.004$).

There are three main smoking cessation pharmacotherapies: varenicline, nicotine replacement therapy (NRT), and buproprion. Each has its specific pharmacologic effects. It is likely that one treatment will work for some people but not others with different genetic backgrounds. Studies of whether the variants in the CHRNA5/A3/B4 gene cluster play a role in smoking cessation has yielded inconsistent conclusions, with some studies demonstrating a significant role of SNPs in this gene cluster in quitting [58, 62–65], whereas others did not [36, 66–68]. Freathy et al. [67] showed strong evidence of an association between rs1051730 and an increased likelihood of continued smoking in pregnancy, supporting a role of genetic factors in influencing smoking cessation. Furthermore, Chen et al. [69, 70], in their two studies, demonstrated that variants in the CHRNA5 gene (rs16969968 or rs16969968–rs680244 haplotype) predicted both ND and smoking cessation. They noted that the high-risk allele of rs16969968 was associated with a lower likelihood of quitting and of cessation failure at end of treatment in the placebo group or the group without any pharmacologic treatment. However, genetic variants did not predict abstinence across active treatment conditions. Thus, Chen et al. [69, 70] suggested that pharmacological cessation treatment might mitigate the genetic risks of cessation difficulty, which might be the explanation for the inconsistent results concerning smoking cessation. Generally speaking, two types of study designs are used in smoking cessation studies. They are either prospective or retrospective, each with different sample selection. This might have different implications. The former identified the genetic risk for smoking cessation, while the latter one placed the emphasis on pharmacologic effects in persons with different genetic backgrounds. There exists a potential limitation for individual study because of differences in sample size, heterogeneity of samples, and analysis approaches, all of which should be taken into consideration in follow-up studies.

Analysis of Rare Variants in the CHRNA5/A3/B4 Gene Cluster

As mentioned above, multiple common variants in the CHRNA5/A3/B4 gene cluster have consistently been found to be significantly associated with ND and smoking-related phenotypes. Among these, a non-synonymous change (rs16969968) in CHRNA5 is the most strongly associated SNP in several GWAS [42, 71]. Additionally, a group of highly correlated SNPs, specifically rs588765, was shown to increase CHRNA5 messenger RNA (mRNA) expression, thus leading to an increased risk of ND [30, 72]. Despite these convincing results, only a small proportion of the variance (~5 %) in smoking-related behaviors can be explained by these SNPs [30]. Rare

variants, generally defined as those having a minor allele frequency of <1 %, constitute another major part of genetic variants other than common ones. Thus, rare variants may well account for the inadequate explanations of the heritability of smoking-related traits, as identified by recent GWAS.

Although rare variants may play a critical role in developing or maintaining ND, the function of these variants in the CHRNA5/A3/B4 gene cluster in the risk of ND has not been intensively investigated [73]. This is, we suspect, largely because their low frequency in populations increases the difficulties in ensuring adequate statistical power. Nevertheless, Wessel et al. [74] recently investigated the contribution of rare variants in nAChR subunit genes to FTND scores in treatment-seeking smokers and observed an association of rare SNPs in CHRNA5 with the FTND score. This finding motivated the interest of Haller and her colleagues in studying rare variants in other nAChR subunit genes in relation to ND. First, the same research team undertook pooled sequencing of the coding and flanking sequences of CHRNA5, CHRNA3, CHRNB4, CHRNA6, and CHRNB3 in African-American (AA) and European-American (EA) ND smokers and in light smokers without symptoms of dependence [75]. They found that rare missense variants at conserved residues in CHRNB4 (for example, rs61737499 and rs12914008) or CHRNA3 (rs8192475 in strong LD with rs12914008) are associated with a lower risk of ND and fewer CPD in both AAs ($p=0.0025$ and $p=6.6\times10^{-5}$, respectively) and EAs ($p=0.023$ and $p=0.021$, respectively) [75].

Using HEK293 cells, Haller et al. examined whether information from this type of functional testing of rare non-synonymous variants in CHRNB4 can significantly improve the association between genotype and phenotype [76]. Consistent with the results from Liang et al. [77], the authors suggested that reduced sensitivity to activation by agonists (nicotine or ACh) results in a higher risk of ND and that, conversely, increased sensitivity reduces the risk. Moreover, an in vivo study has been conducted using models [78] where mice injected in the mHb with lentiviruses carrying the WT β4 subunit or β4 rare missense variants showed aversion to or preference for nicotine, depending on the SNP. For instance, habenular expression of the β4 gain-of-function variant rs61737499 resulted in strong aversion, whereas transduction with the β4 loss-of-function variant rs56235003 failed to induce nicotine aversion. In sum, these functional studies demonstrate the vital role of rare variants in the CHRNA5/A3/B4 gene cluster in smoking-related behaviors.

Functional Studies of the Compelling SNP rs16969968

When the association of a variant with a phenotype of interest is revealed, it represents not only an association with the tested

genetic variant(s), but also an association with untested, highly correlated SNPs that could span several genes on the same chromosome. To understand the molecular mechanism of the CHRNA5/A3/B4 gene cluster associated with ND and/or lung cancer, one needs to determine which SNP might alter biological function. It appears that the most compelling SNP, rs16969968, is likely to be a biological contributor to ND, because it changes an amino acid in the α5 nicotinic receptor protein. The position of the change is in the large cytoplasmic domain adjacent to the conserved amphipathic α-helix, so it is far from the extracellular acetylcholine binding site and unlikely to influence the sensitivity of agonist binding. In such a region, the negatively charged Asp398 might promote Ca^{2+} permeability, whereas Asn398, replaced by an amide group instead of the negatively charged carboxyl group, might inhibit it.

Consistent with this hypothesis, recent studies have demonstrated that the D398N polymorphism affects the function of $(\alpha 4\beta 2)_2\alpha 5$ nAChRs [18, 79]. When the two forms of the human α5 subunit (N398 and D398) were expressed in Xenopus oocytes, using α4 and β2 subunits as a concatamer structure, $(\alpha 4\beta 2)_2\alpha 5$ nAChRs containing the risk allele of α5 associated with increased risk of nicotine addiction exhibited diminished agonist-evoked intracellular calcium response, reduced calcium permeability, as well as enhanced short-term desensitization compared with $(\alpha 4\beta 2)_2\alpha 5$ nAChRs possessing the major allele of α5 [79]. These results were qualitatively similar to those of an earlier study that involved expression in HEK293T cell of human α5 subunits with mouse α4 and β2 subunits [18]. The incorporation of α5 SNP into HEK293T cells transfected with α4β2 cDNA reduced the maximum response to a nicotinic agonist without altering its surface expression. However, these obviously different effects of rs16969968 are seen only on the $(\alpha 4\beta 2)_2\alpha 5$ nAChRs; whether the SNP has a similar effect on the function of $(\alpha 3\beta 4)_2\alpha 5$ nAChRs is unclear.

Morel et al. [25] went a step further, adopting lentiviral re-expression vectors to achieve targeted expression of mutant α5 in the VTA of the brain using a knockin mouse model. It was observed that mice with the SNP rs16969968 in the VTA yielded intermediate behavioral and electrophysiological phenotypes compared with α5 KO mice, suggesting the nonsynonymous α5 variant rs16969968, frequently present in subjects of European descent, exhibits a partial loss-of-function in vivo. This leads to increased nicotine consumption in the self-administration paradigm, thus defining a critical link between this SNP, its expression in VTA DA neurons, and nicotine intake.

Besides rs16969968, there may be a second biologic mechanism in the CHRNA5/A3/B4 gene cluster associated with heavy smoking and ND, including different extents of expression of CHRNA5 mRNA in the brain [80]. Joint statistical analysis of the two loci (or haplotypes) demonstrates that the

amino acid change through SNP rs16969968 and varying CHRNA5 mRNA expression tagged by rs588765 (or rs578776, rs3743078) independently contribute to ND. The risk allele of rs16969968 occurs primarily on the low mRNA expression allele of CHRNA5, whereas the non-risk allele of rs16969968 occurs on both high- and low-expression alleles tagged by rs588765 in CHRNA5. When the non-risk allele occurs against the background of low mRNA expression of CHRNA5, the risk for ND and lung cancer is significantly lower than in persons with higher mRNA expression (Fig. 2). Together, these studies reveal three levels of risk associated with CHRNA5 and at least two distinct mechanisms conferring risk for ND: altered receptor function caused by rs16969968 and variability in CHRNA5 mRNA expression.

However, there is another hypothesis, from a different perspective, to explain the vital function of SNP rs16966698. For example, Hong et al. [81] hypothesized that the smoking variance explained by the allele-modulated circuits was much higher than the smoking variance explained by the genotype alone, making brain circuit measures an intermediate marker for the convergent effects of genes. Thus, the α5 gene variant Asp398Asn is associated with a dorsal anterior cingulated-ventral striatum/extended amygdal circuit, so that the Asn "risk allele" reduced the intrinsic resting functional connectivity strength in this circuit. At the same time, the findings from this work suggest a plausible circuit-level explanation for why rs16969968 and rs578776 represent two independent smoking-related signals in the CHRNA5/A3/B4 gene cluster. The authors of this study distinguished the rs578776-related dACC-thalamus circuit, which appeared sensitive to the "state" of smoking, from the
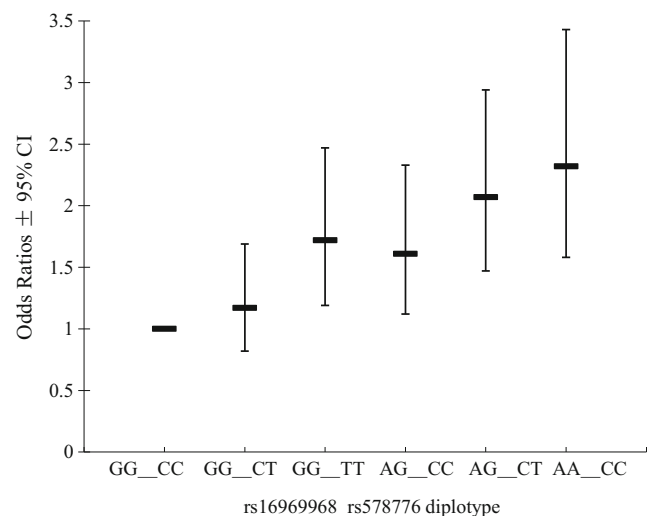

Fig. 2 Association of different rs16969968–rs588765 diplotypes with nicotine dependence. The bars represent odds ratios ±95 % confidence intervals using GG_CC as a reference. A the risk allele of rs16969968, C the low mRNA expression allele of CHRNA5. Adapted from the report by Wang et al. [80], with the permission of Oxford University Press, license number 3416761480752

rs16969968-influenced dACC-ventral striatum circuit, predicting nicotine addiction severity.

From Association to Mechanism: Role of the α5 Subunit

Numerous genetic studies have revealed a strong association between variants in the *CHRNA5/A3/B4* gene cluster and increased vulnerability to ND [17, 50], creating a need to explore the underlying mechanisms. Moreover, to determine the function of the clustered nAChR subunits, KO mice and knockdown rats have been employed primarily because of the lack of receptor agonists and antagonists with selectivity for all three subunits. So far, only α5 and β4 KO mice are available [82–84], and mice that do not express the α3 subunit usually die soon after birth as a result of multi-organ dysfunction [84]. Thus, recent studies mainly focus on the function of α5 and β4 subunits in determining the cause of the high risk of ND, with a special focus on the α5 subunit because of the functional SNP rs16969968.

The α5 nAChR subunit demonstrates a relatively discrete mRNA expression profile in the brain, with the highest densities of expression found in the mHb, which projects almost exclusively to the IPN via the fasciculus retroflexus [85, 86]. Recently, Fowler et al. [24] adopted the α5 KO mouse model (analogous to individuals with reduced α5 receptor function) to examine the underlying mechanism of ND. The α5 KO mice responded far more vigorously than wild-type (WT) mice to nicotine infusions at high doses and consumed significantly more nicotine than their WT littermates when tested under a progressive ratio schedule for reinforcement. Whereas the WT mice tried to control their nicotine intake through intravenous self-administration to achieve a consistent, desired blood concentration, KO mice did not, appearing to consume greater amounts as the dosage increased (Fig. 3). This finding leads to a hypothesis that deficient α5* nAChR signaling attenuates the negative effects of nicotine that limit its intake. Consistent with this result, the same manipulation in rats weakened the aversive effects of higher doses of nicotine but did not alter the reinforcing effects of nicotine on the brain reward system, as measured by nicotine-induced elevations and lowering of intracranial self-stimulation (ICSS) thresholds [24]. These findings are complemented by another study conducted by the same team [87], employing a conditional place preference task to represent the differential effects of nicotine dose on reward in α5 KO and WT mice [88]. Moreover, Fowler et al. showed that the mHb-IPN pathway of the KO mice was far less sensitive to nicotine-induced activation than that in WT mice by using Fos immunoreactivity as a measure of neuronal activation [24]. RNA interference-mediated knockdown of the α5 nAChR subunit in the same rat brain region also resulted in similar responses to nicotine [24]. Intriguingly, virus-mediated re-expression of
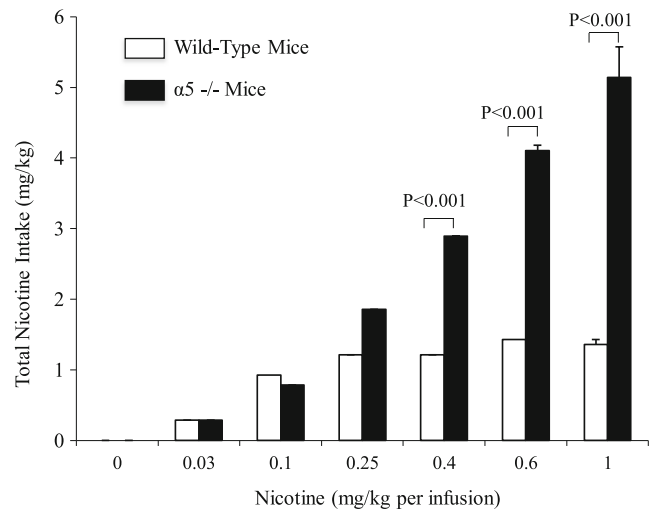

Fig. 3 Increased total nicotine intake (mg/kg) in α5−/− mice compared with WT mice receiving infusions of high doses of nicotine. Data are presented as mean (±SEM) total nicotine intake at each dose. $p < 0.001$ indicates statistically significant differences between these groups at the same nicotine dose. Adapted from the report by Fowler et al. [24], with the permission of Nature Publishing Group, license number 3416820244064

the α5nAChR subunit in the MHb-IPN pathway of the KO mice abolished the increased nicotine intake seen at higher doses of nicotine [24]. Taken together, these findings indicate that the α5 receptor subunit is responsible for transmission of some aversive qualities of nicotine. In other words, nicotine-induced activation of the MHb-IPN pathway by the α5 receptor subunit results in a negative motivational signal that limits further nicotine intake. Hence, disrupted sensitivity of the MHb-IPN tract to nicotine in the α5 KO mice induces greater nicotine intake.

In addition to the α5 nAChR subunit, evidence suggests that β4* nAChRs in the mHb-IPN pathway play a key role in regulating nicotine consumption. For example, Frahm et al. [89] reported that mice overexpressing the β4 subunit as a result of bacterial artificial chromosome (BAC) transgenic technology consumed far less nicotine than their WT counterparts, and this effect could be reversed by lentiviral-mediated expression of the α5 D397N variant in the mHb [89], suggesting that, similar to the α5 nAChR subunit, the β4 subunit regulates sensitivity to the aversive effects of nicotine that control the quantities of drug consumed.

Apart from their role in the aversive effects of nicotine through the mHb-IPN pathway, the α5 and β4 nAChR subunits also have a potential action in nicotine withdrawal. Withdrawal symptoms can be divided into two classes: somatic and affective. The first ones are characterized by increased grooming, scratching, and shaking [90, 91], whereas the latter include primarily depressed mood, anxiety, difficulty concentrating, and so on [90, 92, 93]. The initiation of withdrawal can be precipitated by administration of nicotine antagonists such as mecamylamine during chronic nicotine

exposure. A recent study showed that chronic nicotine-treated β4 KO mice displayed significantly milder somatic withdrawal symptoms than WT mice when the symptoms were precipitated by mecamylamine [94]. Furthermore, α5 KO mice that were dependent on nicotine (delivered through subcutaneously implanted osmotic minipumps) did not show somatic signs of nicotine withdrawal [95]. Considering that β4* and α5* nAChRs are robustly expressed in the mHb-IPN pathway and that mecamylamine was infused directly into either the mHb or the IPN of nicotine-dependent WT mice, the precipitated expression of somatic withdrawal symptoms demonstrates that these two nAChR subunits and perhaps others enriched in the mHb-IPN pathway are critical for the expression of nicotine withdrawal. On the contrary, Fowler et al. [87] concluded that the reward-inhibiting effects of precipitated nicotine withdrawal were not regulated by α5* nAChRs based on the fact that the magnitude to which mecamylamine precipitated elevations of ICSS thresholds was similar in nicotine-dependent WT and KO mice [87]. Interestingly, another study [96] showed that α5* nAChRs are more closely associated with physical signs of nicotine withdrawal than with affective symptoms, because chronic nicotine-treated α5 KO mice still appeared anxious during withdrawal.

Addiction to cigarette smoking depends not only on the attenuating aversion of high doses of nicotine and nicotine withdrawal, as described above, but also on the reinforcing effects of low doses of nicotine, the balance between the rewarding and aversive actions of the drug [90, 92]. Furthermore, although the α5 nAChR subunit is most densely expressed in the mHb-IPN pathway, its expression is also found in many other addiction-relevant brain regions; for instance, a high percentage in the VTA, which underlies the rewarding and addictive properties of drugs of abuse through the dopaminergic (DAergic) neurons [97]. Consequently, the α5* nAChRs are subjected to the same action in the VTA that explains their role in ND. However, many studies trying to identify the role of the α5 receptor subunit in the mHb-IPN pathway failed to find an effect in the VTA, especially in the dopaminergic neurons [24, 87]. There was a first report that comprehensively analyzed the role of the α5 nAChR subunit in the VTA DA system [25]. This study investigated the reinforcing effects of nicotine in drug-naive α5 KO mice by using an acute intravenous nicotine self-administration task and ex vivo and in vivo electrophysiological recording of nicotine-elicited DA cell activation. The fact that α5 KO mice, compared with WT mice, exhibited decreased sensitivity of the DAergic system and a dramatic shift to high nicotine doses in an acute nicotine injection paradigm [25] suggested a crucial role of α5* nAChRs in determining the minimum nicotine dose necessary for DA activation and thus nicotine reinforcement (Fig. 4). In addition, normal responses like those in WT mice were restored in KO mice by generalized lentiviral-mediated re-expression of the α5 subunit in all VTA

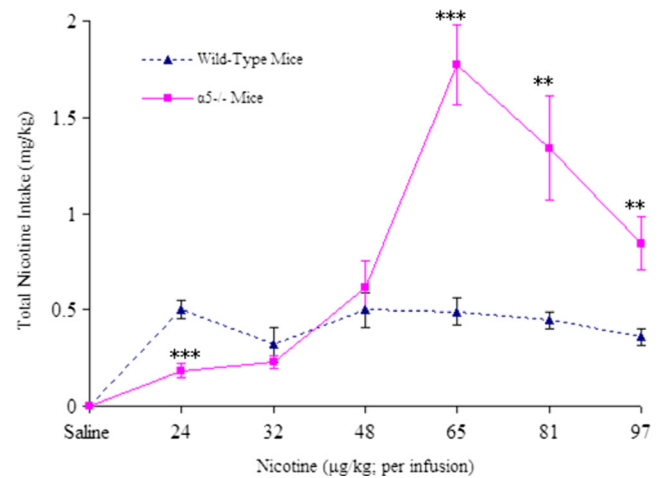**Fig. 4** Crucial role of α5* nAChRs in intravenous self-administration task (IVSA). α5−/− mice exhibited a decreased sensitivity of the DA neurons and a dramatic shift to high nicotine doses compared with WT mice. Data are presented as mean (±SEM) total nicotine intake at each dose. ***$p<0.001$; **$p<0.01$. Adapted from the report by Morel et al. [25], with the permission of Nature Publishing Group, license number 3416821041833

cells or targeted to VTA DA cells specifically [25]. These findings have defined novel, largely unexpected roles for the α5 nAChR subunit in reinforcing the effects of nicotine, although it acts only as an accessory subunit instead of contributing to the nicotine binding site. This aspect of the research may broaden our horizons in understanding the underling mechanisms of the *CHRNA5/A3/B4* gene cluster in the development of ND, although independent verification of the findings is still lacking.

Conclusions and Future Research

Cigarette smoking continues to be a major health threat worldwide, underscoring the need to fully understand the etiology of ND. Research has implicated variants in the *CHRNA5/A3/B4* gene cluster on chromosome 15 in the development of ND [17, 18, 20, 34]. There is now a compelling body of evidence linking SNPs rs16969968 (or its strongly linked SNPs) and rs578776 (or rs588765) to smoking-related phenotypes [17–20, 34]. Joint statistical analyses of the two loci mentioned above suggest the existence of two independent molecular mechanisms in ND. One is the amino acid change through SNP rs16969968, and another is differing degrees of *CHRNA5* mRNA expression tagged by rs588765 (or rs578776, rs3743078) [80]. However, these findings reveal only a small portion of both common and rare variants in the *CHRNA5/A3/B4* cluster. Thus, additional loci associated with smoking-related phenotypes await discovery. In particular, despite its difficulty, much attention should be paid to studies of rare variants in this gene region in order to understand in depth the genetics of ND.

There still is some controversy as to the relation between the implicated SNPs and lung cancer, although the findings from GWAS are robust [55–57]. Whether this association is direct or merely a byproduct of ND must be investigated further. Because there have been no specific pharmacological reagents for the α5, α3, or β4 nAChR subunits that are useful in elucidating such complicated relations, design of highly specific nAChRs ligands is of prime importance. Alternatively, knockin mouse model studies may directly examine the effects of variants given a constant carcinogen exposure. In other words, if, for example, SNP rs16969968 can be inserted into mice while ensuring that other conditions remain the same, the difference between the two groups of mice would be only in this SNP. Supposing that there is a difference in lung cancer between the two groups of mice, we can conclude that rs16969968 acts directly in the development of lung cancer. However, if not, we are more willing to believe that the SNP plays an indirect role.

As with the rapid development of the large-scale GWAS, extensive genomic information concerning ND is now available. This lays emphasis on the urgency of understanding the biological mechanisms of how α5, α3, and β4 nAChR subunits modulate smoking-related behaviors, which presents both opportunities and challenges. An important means to tease out the functional role of these receptor subunit genes in the neurobiology of ND is through genetic engineering technologies. Significant progress has been made in the past few years by using both in vitro and in vivo models, highlighting the importance of the α5 nAChR subunit in regulating ND. However, these functional studies so far reveal only a critical role of the α5 subunit in controlling the aversive and withdrawal effects of nicotine. How the α3 or β4 nAChR subunits function in ND has not been clarified yet, primarily because of the smaller number of functional studies of these two subunits. Even though there are a few studies suggesting a role of the α5 subunit in the rewarding effect of nicotine, most of them remain to be validated in independent studies. Thus, this part of research is in its early stages, and more relevant studies are greatly needed so as to fully understand the underlying mechanisms of ND. In addition, as discussed above, there exists a significant interaction between SNPs or haplotypes in the *CHRNA5/A3/B4* gene cluster and the success of cessation measures. Those with the high-risk SNPs or haplotypes appear more biologically predisposed to having difficulty quitting without pharmacologic treatment, a problem that may be ameliorated by effective pharmacologic treatment. Thus, identification of molecular mechanisms underlying ND and responsiveness to pharmacologic treatment for ND will improve the development of novel, tailored smoking cessation therapies.

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