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Genetic Variability in Nicotinic Acetylcholine Receptors and Nicotine Addiction: Converging Evidence from Human and Animal Research

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Abstract

Tobacco smoking is a leading preventable cause of death in the United States and produces a major health and economic burden. Although the majority of smokers want to quit, few are successful. These data highlight the need for additional research into the neurobiology of tobacco dependence. Addiction to nicotine, the main psychoactive component of tobacco, is influenced by multiple factors that include individual differences in genetic makeup. Twin studies have demonstrated that genetic factors can influence vulnerability to nicotine addiction, and subsequent research has identified genes that may alter sensitivity to nicotine. In humans, genome-wide and candidate gene association studies have demonstrated that genes encoding nicotinic acetylcholine receptor (nAChR) proteins are associated with multiple smoking phenotypes. Similarly, research in mice has provided evidence that naturally occurring variability in nAChR genes is associated with changes in nicotine sensitivity. Furthermore, the use of genetic knockout mice has allowed researchers to determine the nAChR genes that mediate the effects of nicotine, whereas research with knockin mice has demonstrated that changes to nAChR genes can dramatically alter nicotine sensitivity. This review will examine the genetic factors that alter susceptibility to nicotine addiction, with an emphasis on the genes that encode nAChR proteins.

Keywords

Genetics; Nicotine; Addiction; Withdrawal; Acetylcholine; Mice

Introduction

The negative health and economic consequences of tobacco use have been well established [17,137]; however, tobacco smoking causes more deaths worldwide per year than all other drugs combined [136]. In the United States, the majority of smokers (68%) report that they want to quit smoking, but only 5% are successful [16,116]. Although pharmacotherapeutics for smoking cessation such as nicotine replacement therapy, bupropion, and varenicline are currently available, these drugs only produce a 1.5 to 3 fold increase in the likelihood of successfully quitting [14,72,153]. Given that the rates for successfully quitting smoking are

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low even with pharmacotherapeutic intervention, there is a clear need for additional research into the neurobiology of tobacco dependence.

Addiction to nicotine, the main psychoactive component of tobacco [4], produces numerous changes to behavioral and neural functioning [76,131] that can be modulated by environmental, developmental, and genetic factors [3,18,67]. Research has demonstrated that having family or peers that smoke will increase the likelihood of ever smoking [62,63]. Furthermore, epidemiological studies suggest that adolescents are more vulnerable to nicotine addiction: 88% of smokers tried their first cigarette before age 18, and the development of daily cigarette smoking typically occurs before age 25 [10,41]. There is also considerable evidence that genetics influence several smoking-related behaviors. Twin studies have been used extensively to estimate genetic contributions to smoking-related behaviors, such as persistent or current smoking (i.e. long-term continuous tobacco use), number of cigarettes smoked daily, and diagnosed nicotine dependence. Heritability estimates for these smoking phenotypes range from 39 – 82% for persistent smoking [93,100,101,175], 45 – 86% for number of cigarettes smoked daily [65,83,164,178], and 31 – 75% for nicotine dependence [80,115,176,179]. In addition, genetic factors also influence nicotine withdrawal symptoms and failed smoking cessation: heritability estimates for nicotine withdrawal symptoms range from 29 – 53%, whereas estimates for smoking cessation are 51 – 54% [91,127,188,189]. Overall, these data suggest that genetics play an important role in smoking-related behaviors, and therefore an improved understanding of the genetics of nicotine addiction will aid in the development of effective smoking cessation treatments.

In recent years, research has demonstrated that genes that encode nicotinic acetylcholine receptor (nAChR) proteins may play an important role in susceptibility to nicotine addiction. Genome-wide linkage analyses have identified several loci across the human genome that may contain genes that increase vulnerability to a given smoking-related behavior or symptom, such as nicotine dependence or the number of cigarettes smoked. nAChR subunit genes have been found within or near these loci, suggesting that variability in these genes may alter sensitivity to nicotine addiction. Furthermore, candidate gene association studies have demonstrated that variability in genes that encode nAChR subunits are associated with multiple smoking phenotypes. Animal research has also provided insight into genetics of nicotine addiction: numerous studies have evaluated the effects of acute nicotine, chronic nicotine, and nicotine withdrawal in mice with genetically modified nAChR subunits, and several studies have investigated whether naturally occurring variability in nAChR subunit genes can influence sensitivity to the effects of nicotine. Thus, research in both humans and animals suggest that genes involved in cholinergic functioning play an important role in altering risk for nicotine addiction, and a more complete understanding of how these genes influence nicotine sensitivity may lead to development of more effective therapeutics for nicotine addiction.

Genome-wide Linkage Analyses

A genome-wide linkage study is an unbiased search for regions across the entire human genome that are strongly associated with a given phenotype (e.g. the number of cigarettes smoked daily). Such studies are ideally conducted using hundreds of large families that contain both smokers and non-smokers, and loci that are similar in affected relatives and dissimilar with non-affected relatives are thought to contain genes that may increase vulnerability to smoking-related behaviors. Associations between genetic variability and a smoking phenotype are plotted as peaks at specific loci across the genome, and the magnitude of the effect is typically determined by a logarithm of the odds (LOD) score. Given that loci identified in linkage analyses are often not replicated in subsequent investigations, Lander and Kruglyak [87] have developed guidelines to evaluate the strength of a reported linkage. The criterion for “significant linkage” depends upon the design of the genome-wide linkage study, and is defined

as LOD scores greater than 3.3 – 3.8 or a p value less than 4.9×10^{-5} – 1.3×10^{-5} , whereas the criterion for “suggestive linkage” also depends upon the study design, and is defined as LOD scores greater than 1.9 – 2.4 or a p value less than 1.7×10^{-3} – 4.2×10^{-4} [87]. For example, in a linkage study that examines allele sharing between sibling pairs, the criterion for significant linkage is defined as an LOD score that is greater than 3.6 or a $p < 2 \times 10^{-5}$, whereas “suggestive linkage” is defined as LOD scores that range from 2.2 – 3.6, or a $p < 7 \times 10^{-4}$.

Linkage studies have evaluated several smoking phenotypes, such as smoking initiation [120,178], the number of cigarettes smoked daily (smoking quantity) [92,96,95,120,165,182], nicotine dependence [50,96,95,98,165], and withdrawal severity [165], and overall, one or more regions in nearly all chromosomes have been implicated in smoking-related behaviors. Furthermore, nAChR subunit genes have been found within some of the loci identified in linkage studies, suggesting that these genes may warrant further investigation. Using the criteria established by Lander and Kruglyak [87], suggestive linkage has been found for smoking initiation on chromosome 6 in a sample of Dutch twins [178]; other research has implicated chromosomes 16 and 20, but p values were below the criteria for suggestive linkage [120]. In addition, significant linkage to smoking quantity has been found in regions located on chromosomes 1 and 10–12, whereas chromosomes 3–5, 7–9, 13, 16, 17, 18, and 20 exhibit suggestive linkage to smoking quantity [92,96,95,120,165,182]. Several linkage studies have also evaluated nicotine dependence (defined using the Fagerström Test for Nicotine Dependence; FTND, or defined using the DSM-IV), and suggestive linkage for nicotine dependence has been found in regions of chromosomes 3–11, and 20 [50,96,95,98,165]. Additionally, the genes that encode the $\alpha 2$ and $\alpha 10$ nAChR subunits (*CHRNA2* and *CHRNA10*, respectively) were found within the loci associated with nicotine dependence, suggesting that these genes may influence susceptibility to nicotine addiction [98,165]. Finally, Swan and colleagues [165] reported suggestive linkage between a region on chromosome 6 and the severity of nicotine withdrawal, and significant linkage to short term quitting (defined as quitting smoking for > 1 month but < 1 year) was found on chromosome 16.

Overall, the data from genome-wide linkage studies reveal that nicotine addiction is a complex disorder that is modulated by numerous genes distributed throughout the human genome, as well as by the interactions between these genes. However, similar to investigations of other complex disorders, few of the loci implicated by linkage studies have been replicated in subsequent research. Several factors likely contribute to this inconsistency across studies. For example, the loci that are implicated in a given smoking-related behavior may vary as function of ethnicity or gender, and it may be difficult to detect these differences if the subject pool is heterogeneous. In support, research has identified different loci for nicotine dependence in samples with European American or African American ancestry [50,96], and combining both samples decreased the ability to detect some loci [96], which suggests that genetically heterogeneous samples may mask the detection of regions that are ethnicity-specific. Other factors that may limit the replicability of linkage studies are differences in defining phenotypes, the statistical methods used, and differences in sample size. Despite these limitations, genome-wide linkage studies have provided a useful starting point for the identification of genes that may influence smoking phenotypes. Several genes that encode receptors are located within or near the regions implicated by linkage studies, including the *CHRNA2* and *CHRNA10* nAChR subunit genes, dopamine receptors 1 and 4 (*DRD1* and *DRD4*, respectively), the μ_1 opioid receptor (*OPRM1*), and the GABA_B receptor subunit 2 (*GABAB2*) [95,98,165,180]. Thus, numerous candidate and genome-wide gene association studies have evaluated whether variations in these genes are related to smoking phenotypes.

Nicotinic Acetylcholine Receptors: Candidate Gene and Genome-Wide Association Studies

The effects of nicotine are primarily mediated by its actions on nicotinic acetylcholine receptors (nAChR); thus, single nucleotide polymorphisms (SNPs) in genes that encode nAChRs are likely to modulate the effects of nicotine. nAChRs are ligand-gated cation channels that are composed of 5 subunits, and can exist either as homopentamers or heteropentamers (i.e. 5 identical subunits or 5 non-identical subunits, respectively) [68,90]. Currently, 12 neuronal and 5 muscle nAChR subunits have been identified (Neuronal: $\alpha 2 - \alpha 10$, $\beta 2 - \beta 4$; Muscle: $\alpha 1$, $\beta 1$, γ , δ , and ϵ), and research has demonstrated that the $\alpha 4$, $\beta 2$, and $\alpha 7$ nAChR subunits are the most abundantly expressed in the brain [68,90]. nAChR subtypes, such as the $\alpha 4\beta 2^{*1}$ and $\alpha 7$ receptors, exhibit functional diversity in their rate of desensitization, binding affinity, and cation permeability [25,46,125]. For instance, $\alpha 4\beta 2^{*}$ nAChR subtypes bind with high affinity to nicotine and desensitize at a slow rate, whereas $\alpha 7$ subtypes have a lower affinity for nicotine and desensitize rapidly [25,46]. Thus, the various behavioral and neural effects of nicotine are likely to be mediated by different nAChR subtypes.

Several candidate gene association studies have investigated whether polymorphisms in the genes that encode neuronal nAChR subunits (*CHRNA2* – *CHRNA10*, and *CHRNA2* – *CHRNA4*) are associated with increased or decreased vulnerability to smoking-related behaviors. In candidate gene studies, researchers determine whether one or more alleles in a gene of interest appear more frequently in smokers than in non-smokers. There are several advantages to using this approach. Unlike genome-wide linkage studies, association studies can be conducted using a case-control design (comparing unrelated smokers to non-smokers) or with family-based designs (comparing allele sharing between smokers and their parents or between siblings). Additionally, candidate gene studies may be a more effective approach for identifying genes that produce small changes in risk for smoking phenotypes [138]. One limitation of using the candidate gene approach is that the genes selected for investigation typically depend upon what is currently known about nicotine addiction, and other genes that may also play an influential role can be overlooked. An additional limitation to candidate gene association studies is that false positive results can occur in case-control studies because smoker and non-smoker groups may consist of individuals from diverse genetic backgrounds (i.e. population stratification), and therefore researchers must evaluate the genetic similarity of both groups to control for this issue [161].

Recent advances in genotyping, along with development of databases of genetic variability in the human genome (e.g. the dbSNP database), have led to the development of the genome-wide association studies, which can evaluate thousands of SNPs distributed across the human genome [66,184]. Similar to a candidate gene association study, allele frequencies in unrelated smokers are compared to controls in a genome-wide association study. However, the focus in a genome-wide association study is not alleles in a particular gene but instead is an unbiased assessment of allele frequencies that are distributed throughout the entire human genome [66,184]. The unbiased selection of alleles across the human genome is one major advantage to this approach: genes that have been overlooked by candidate gene studies may be identified as playing a role in altering risk for nicotine addiction. However, several issues must be taken into consideration when implementing a genome-wide association study. For instance, it has been recommended that a p -value of 1×10^{-6} be used because of the numerous statistical tests used in genome-wide association studies [184]. Furthermore, a large subject pool is needed so that there is sufficient statistical power to detect gene variants that are associated with a phenotype, and the potential for population stratification between cases and controls must also

¹An “*” indicates that other nAChR subunits may combine with the $\alpha 4$ and $\beta 2$ subunits to form receptors.

be assessed [66]. Finally, the independent replication of findings in subsequent genome-wide association studies can verify that the genes identified are not false positives [66,184].

The $\alpha 4$ nAChR subunit (CHRNA4)

Candidate gene association studies have yielded evidence that several variants of *CHRNA4* are protective against smoking-related behaviors (see Table 1 for a summary of candidate gene association studies). A study using a sample of Chinese men identified 2 SNPs (rs1044396 and rs1044397) in exon 5 of *CHRNA4* that were associated with reduced risk for nicotine dependence [45]. Both SNPs were synonymous² and a haplotype of 6 SNPs that included both of the protective variants also was associated with reduced risk for nicotine dependence. Additionally, four SNPs and a haplotype in *CHRNA4* have been identified in European Americans and African Americans that were associated with smoking-related behaviors, but the effects varied as a function of ethnicity or gender [94]. Specifically, one synonymous SNP (rs1044396) located in exon 5 was associated with smoking quantity in European Americans, whereas an additional SNP (rs3787137) located in intron 5 was associated with smoking quantity in European American men only. Furthermore, a SNP (rs2236196) located in the 3' untranslated region (UTR) was associated with nicotine dependence in African American women, and a fourth SNP (rs2273504) was associated with nicotine dependence in African and European American women. A haplotype that consisted of 3 SNPs (including both protective variants of rs2273504 and rs2236196) was found to reduce risk for nicotine dependence in African American women [94].

Hutchison and colleagues [75] have identified SNPs in *CHRNA4* in smokers that were associated with subjective responses to smoking. Following an 8 hour abstinence period, participants smoked a cigarette and rated the effects of smoking on 4 dimensions (physical effects, cognitive effects, rush or high, and reward); participants with a heterozygous genotype at SNP rs2236196 (i.e. possessed both variants of this SNP) exhibited an increased sensitivity to all 4 subjective measures of smoking. Additional analyses of this SNP revealed that nicotine nasal spray was a more effective smoking cessation aid than a transdermal nicotine patch in individuals that were heterozygous. Furthermore, an additional SNP (rs6122429) was identified that was associated with increased sensitivity to the rewarding effects of smoking [75]. Together, the results of these studies suggest that variation in *CHRNA4* can influence several smoking phenotypes, including the physical, cognitive, and rewarding properties of smoking. However, it is also evident that the effects of some SNPs or haplotypes may depend upon ethnicity or gender, whereas other SNPs may have similar effects across all groups. For instance, Li and colleagues [94] identified different SNPs in African vs. European Americans that were associated with nicotine dependence, but other SNPs such as rs2236196 have been associated with smoking phenotypes in several ethnic groups [75].

The $\beta 2$ nAChR subunit (CHRNA2)

Given that $\alpha 4\beta 2^*$ nAChRs are the predominant high-affinity binding sites for nicotine in the brain [47], and that research using genetic knockout (KO) mice has revealed that mice lacking the $\beta 2$ nAChR subunit have almost no high-affinity binding for nicotine [129], several studies have investigated whether polymorphisms in *CHRNA2* are related to smoking phenotypes. In contrast to research on *CHRNA4*, evidence for the role of *CHRNA2* in smoking phenotypes has been less robust; several studies have found no relation between *CHRNA2* polymorphisms and nicotine dependence [45,94,99,154]. However, Greenbaum and colleagues [61] identified one SNP (rs2072660) located in the 3' UTR, along with a 5 SNP haplotype, that was associated

²In a synonymous SNP, the substitution of a nucleotide produces no changes in amino acid sequence, whereas in a non-synonymous SNP, a nucleotide substitution will change the amino acid sequence of the protein.

with reduced risk for smoking initiation in Israeli females. Furthermore, a recent study investigated the relation between *CHRNA2* and initial subjective responses to smoking (e.g. feeling dizzy or nauseous after smoking) and identified one SNP (rs2072658) located upstream of the *CHRNA2* gene that was associated with an increased response to the negative effects of smoking, whereas another SNP (rs2072660) located in the 3' UTR was associated with a reduced subjective response to the negative effects of smoking [40]. Thus, the results from these studies suggest that polymorphisms in *CHRNA2* may also modulate smoking-related behaviors. Given that several studies were unable to find any relation between *CHRNA2* and smoking phenotypes, it is possible that its effect on smoking-related behaviors is small or may depend upon the expression of other genes. For example, *CHRNA2* may interact with other genes such as *CHRNA4* to influence nicotine dependence. Therefore, future research should investigate possible epistatic effects between *CHRNA2* and other genes.

The *CHRNA3-CHRNA6* and *CHRNA5-CHRNA3-CHRNA4* Gene Clusters

Research using genome-wide association and candidate gene approaches has demonstrated that polymorphisms in the *CHRNA3-CHRNA6* gene cluster on chromosome 8 are strongly associated with nicotine dependence. Bierut and colleagues [8] evaluated 31,960 SNPs for associations with nicotine dependence (measured using the FTND); 2 SNPs in the *CHRNA3* gene (rs13277254 and rs6474413) were associated with risk for nicotine dependence. This finding was supported by a candidate gene association study of 348 genes that was conducted simultaneously by the same group: 2 SNPs located in the 5' promoter region of *CHRNA3* (rs10958726 and rs6474413), 2 synonymous SNPs located in exon 5 of *CHRNA3* (rs4953 and rs4952) and 1 SNP in *CHRNA6* (rs2304297) were strongly associated with risk for nicotine dependence [142]. Furthermore, an additional candidate gene association study identified four SNPs in *CHRNA3* (rs4950, rs13280604, hCV25772398, rs4953) that were associated with adverse (e.g. feeling depressed or paranoid after smoking) and positive (e.g. feeling energetic or sociable after smoking) subjective responses to smoking, and these effects were replicated for 2 SNPs (rs4950 and rs13280604) in a separate sample [192]. Additionally, one SNP in *CHRNA6* (rs2304297) was associated with positive subjective responses to smoking in one sample. Overall, these data provide strong evidence that SNPs in *CHRNA3* and possibly *CHRNA6* are related to multiple smoking phenotypes. Given that the $\alpha 6$ and $\beta 3$ subunits are highly expressed in the ventral tegmental area (VTA) and the nucleus accumbens (NAC), which are brain areas implicated in the rewarding effects of nicotine [82,89], additional research should evaluate whether polymorphisms in *CHRNA3* and *CHRNA6* are related to the rewarding effects of smoking.

Candidate gene association studies investigating the *CHRNA5-CHRNA3-CHRNA4* gene cluster located on chromosome 15 have found evidence that variability in this gene cluster is associated with risk for several smoking phenotypes. Saccone and colleagues [142] have reported strong associations between nicotine dependence and the *CHRNA5-CHRNA3-CHRNA4* gene cluster. Specifically, 3 SNPs in *CHRNA5* (rs16969968, rs637137, and rs684513), 3 SNPs in *CHRNA3* (rs578776, rs1051730, and rs3743078) and 1 SNP in *CHRNA4* (rs3813567) were related to risk for nicotine dependence. Additional research has demonstrated associations between the *CHRNA5-CHRNA3-CHRNA4* gene cluster and smoking initiation (e.g. smoking at least once a month); 1 SNPs in *CHRNA5* (rs514743), 1 SNP in *CHRNA4* (rs1948), and an intergenic SNP located between *CHRNA3* and *CHRNA4* (rs8023462) were associated with an earlier age of smoking initiation and were replicated in an independent sample [150].

Finally, several genome-wide association studies have demonstrated that polymorphisms in the *CHRNA5-CHRNA3-CHRNA4* gene cluster may influence smoking-related behaviors and alter risk for lung cancer. Berrettini and colleagues [6] evaluated smoking quantity in two

independent samples of European ancestry, and identified a SNP in *CHRNA3* (rs6495308) that was associated with increased smoking quantity in both samples. An additional SNP in *CHRNA3* (rs1317286) was related to increased smoking quantity in a third sample. These SNPs, along with those identified by Saccone and colleagues [142], belong to a common *CHRNA5-CHRNA3* haplotype that is associated with risk for smoking quantity. Three independent genome-wide association studies have also linked the *CHRNA5-CHRNA3-CHRNA4* gene cluster to risk for lung cancer, smoking quantity, and nicotine dependence [1, 74, 171]. A SNP in *CHRNA3* (rs1051730) that is in linkage disequilibrium with other SNPs in the *CHRNA5-CHRNA3-CHRNA4* gene cluster [142] was associated with smoking quantity, nicotine dependence, risk for peripheral arterial disease [171], and risk for lung cancer [1, 74, 171]. Furthermore, Hung and colleagues [74] identified a non-synonymous SNP in *CHRNA5* (rs16969968) that was associated with risk for lung cancer, and this finding was replicated in five independent studies. Although these data suggest that the *CHRNA5-CHRNA3-CHRNA4* gene cluster is directly associated with risk for lung cancer, it is possible that this gene cluster alters risk for nicotine dependence, and subsequent tobacco smoking increases risk for lung cancer. Some evidence suggests that the link between the *CHRNA5-CHRNA3-CHRNA4* gene cluster and lung cancer is independent from any association between the gene cluster and nicotine dependence [1, 74], whereas other research indicates that risk for lung cancer may be indirectly modulated by an association between the *CHRNA5-CHRNA3-CHRNA4* gene cluster and nicotine dependence [171]. Together, these data suggest that polymorphisms in the *CHRNA5-CHRNA3-CHRNA4* gene cluster may influence several smoking phenotypes and alter risk for lung cancer, but it is unclear whether these effects are mediated by SNPs in one or more of the genes in this cluster.

The reviewed research from candidate gene and genome-wide association studies has demonstrated that genetic variability in several nAChR subunit genes (*CHRNA3-CHRNA6*, *CHRNA2-CHRNA4*) can influence nicotine dependence, as measured by a variety of smoking phenotypes. It is also clear that some nAChR subunit genes may have a greater effect on nicotine dependence than others, and that the effects of a given nAChR subunit gene may depend upon factors such as ethnicity and gender. However, there is much that remains unknown about the role of nAChR subunit genes in nicotine addiction. To date, there are several nAChR subunit genes that have not been directly investigated, including the *CHRNA2* and *CHRNA10* genes which have been identified as possible candidate genes in genome-wide linkage scans [98, 165]. Additionally, future research should assess the relationship between nAChR subunit genes and nicotine dependence with a more comprehensive selection of smoking phenotypes, including measures of nicotine withdrawal (e.g. number of withdrawal symptoms, withdrawal severity) and the efficacy of pharmacotherapeutic treatments for smoking cessation. In support, it has been shown that variations in *CHRNA4* are associated with smoking cessation outcomes [75], and it is possible that polymorphisms in nAChR subunit genes may modulate the effects of nicotine replacement therapy and varenicline (a $\alpha 4\beta 2^*$ partial agonist and $\alpha 7$ nAChR full agonist) [21, 118] on smoking cessation. Furthermore, given that the protein products of these genes are known to interact with each other to form receptors, it is possible that interactions between nAChR subunit genes may have a greater influence on smoking phenotypes than an individual nAChR subunit gene; thus, additional research should investigate possible gene-gene interactions between nAChR subunit genes.

Nicotinic Acetylcholine Receptors: Animal Research

Nicotine addiction is a complex disorder that is composed of numerous changes to behavior and physiology and can be characterized by several stages such as experimentation, regular use, nicotine dependence, attempts to quit/smoking cessation, and relapse [112]. Several differences exist between humans and other animals, such as the physiologically relevant range of nicotine doses used and the rate of nicotine metabolism [111], and some aspects of nicotine

addiction in humans cannot be easily assessed by animal research (e.g. relapse). Despite these limitations, animal research has modeled several effects of acute and chronic nicotine that relate to the effects of nicotine in humans. Similar to other drugs of abuse, nicotine increases dopamine (DA) release in the nucleus accumbens, and the effects of nicotine on the mesolimbic dopaminergic pathway is thought to mediate the rewarding properties of nicotine [42]. Several behavioral paradigms, such as nicotine self-administration and nicotine conditioned place preference (CPP) have been used to model the rewarding effects of nicotine in animals [139, 155]. In nicotine self-administration studies, rodents learn to respond on a lever for nicotine infusions, or receive oral nicotine in a two bottle choice procedure (nicotine-treated water vs. untreated water), whereas subjects in a nicotine CPP procedure learn to prefer a chamber paired with nicotine administration over another chamber that was paired with saline.

In addition to changes in reward, evidence from both human and animal research has demonstrated that nicotine alters cognitive processes, including associative learning [35,37, 48,58,88]. For example, cravings for nicotine were increased following the presentation of previously neutral environmental cues that were associated with smoking [88]. Similarly, withdrawn smokers and non-withdrawn smokers that were exposed to smoking-related stimuli reported increases in cravings [39,48,49]. Research has also demonstrated that contextual cues (i.e. cues that indicate smoking availability) can increase cravings for smoking [38,37,170, 169]. Consistent with these data, research in mice has demonstrated that nicotine alters contextual learning. In mice trained to form an association between an auditory conditioned stimulus (CS) and a footshock unconditioned stimulus (US; cued conditioning), and an association between the training context CS and the US (contextual conditioning), acute nicotine enhances contextual conditioning, but has no effect on cued conditioning [30,32,31, 56,55,186]. However, contextual conditioning is not altered by a dose of chronic nicotine that produces the same plasma nicotine levels as the acute dose that produces enhancement, suggesting the development of tolerance [30]. It is important to note that these plasma nicotine levels were within the range observed in smokers [5,64]. Additionally, withdrawal from chronic nicotine disrupts contextual but not cued conditioning [2,33,30,132,133], and this impairment in contextual learning can be ameliorated by nicotine replacement and by the smoking cessation drugs bupropion and varenicline [30,132,135]. Together, these data suggest that associations between stimuli and the effects of nicotine in humans may contribute to nicotine addiction, and these effects can be modeled in mice by studying the effects of nicotine on contextual learning.

Nicotine withdrawal in humans consists of multiple symptoms, such as insomnia, anxiety, increased appetite, and cravings [73,71]. Furthermore, nicotine withdrawal produces changes in cognition, including the disruption of attention [78,141], difficulty in concentration [70, 71], and learning and memory deficits [78,79,117]. Many of these nicotine withdrawal phenotypes have been studied in animals. For instance, some research has evaluated somatic symptoms (e.g. increased grooming, scratching, or shaking) in rats or mice that occur following spontaneous withdrawal or nAChR antagonist-precipitated withdrawal [28,77,103,102]. Additionally, mice withdrawn from chronic nicotine have been evaluated using the elevated plus maze, in which the percentage of time spent on the open arms of the maze serves as a measure of anxiety [28,77]. Mice withdrawn from nicotine spend less time on the open arms relative to saline controls, indicating increased anxiety [28,77]. Furthermore, nicotine withdrawal-related deficits in contextual learning have been evaluated in mice [33,30,132, 133]. Withdrawal-related decreases in the brain reward system in humans has been modeled using the intracranial self stimulation (ICSS) procedure, in which rats press a lever to deliver electrical stimulation to reward-related brain areas. Nicotine withdrawal increases ICSS threshold [26,81,156]. Thus, several nicotine withdrawal symptoms reported in humans have been modeled and studied in rodents.

Although the development of a perfect model of nicotine addiction is not possible, multiple animal models of nicotine addiction have been designed that correspond to some of the endophenotypes observed in humans. Furthermore, several, but not all, of the behavioral procedures that model nicotine addiction have been used to investigate the genetic factors that influence sensitivity to nicotine. In the sections that follow, we will discuss research that has utilized these behavioral procedures to demonstrate that nAChR subunit genes play an important role in regulating the effects of nicotine.

The Effects of Nicotine in Inbred Rodent Strains

The genetic factors that influence the effects of nicotine have been investigated in rodents using two different approaches. In order to determine whether naturally occurring genetic variability can modulate nicotine sensitivity, researchers have characterized the effects of nicotine in two or more inbred strains of rodents. By definition, inbred strains are genetically identical after 20 consecutive generations of brother/sister mating [24]; thus, differences between strains reveal genetic influences on sensitivity to nicotine. However, some genetic differences between members of an inbred strain have been reported. For example, copy number variations have been identified in C57BL/6J mice that can alter gene expression [185]. In addition, genetic drift can produce differences in behavior between substrains of an inbred mouse strain that are bred by separate sources (e.g. C57BL/6J vs. C57BL/6Jbg) [158].

The use of genetically modified mice is an additional approach that can be utilized to study the genes that are required for the effects of nicotine. One advantage to this approach is that the use of KO mice can pinpoint the genes that mediate the effects of nicotine. However, the deletion of a gene does not reveal whether variability in this gene can alter sensitivity to nicotine. The generation of knockin (KI) mice that have had a hypersensitive or hyposensitive nAChR subunit inserted into the mouse genome can be used to investigate whether variability in nAChR subunit genes can modulate nicotine sensitivity, but this artificial change in the functional properties of the subunit may not reflect what occurs naturally. Furthermore, it can be difficult to establish whether the effects observed in genetically modified mice are due to the alteration of a gene, or if they result from compensatory changes that occur during development. Despite the limitations of these two approaches, animal research has yielded evidence that genetic variability in nAChR subunits can alter sensitivity to nicotine.

A growing body of research has characterized the effects of nicotine in several strains of inbred rats and has revealed differences in sensitivity to nicotine across multiple strains. For instance, several differences in the rewarding properties of nicotine have been characterized in the Lewis and Fischer 344 inbred rat strains. Lewis rats can learn to self-administer nicotine in an unlimited access procedure but not when there is limited access to nicotine, whereas Fischer 344 rats do not self-administer nicotine in either procedure [12,151]. Furthermore, Lewis rats can rapidly learn nicotine CPP when lower doses of nicotine are used, whereas Fischer 344 rats require more trials to acquire nicotine CPP at lower doses and can only learn the procedure rapidly when a high dose of nicotine is used [69,128]. In addition to these differences in the behavioral effects of nicotine, Sziraki and colleagues [166] have demonstrated that nicotine-induced increases in DA release in the nucleus accumbens is greater in Lewis rats than in Fischer 344 rats, and plasma nicotine was cleared faster in Lewis rats. Together, these data suggest that Lewis rats are more sensitive to the rewarding properties of nicotine, and may have increased vulnerability for nicotine dependence. Given that research with other drugs of abuse have demonstrated that Lewis rats acquire cocaine and morphine self-administration more rapidly than Fischer 344 rats [84,107], it is possible that Lewis rats have a greater generalized sensitivity for drug reinforcement. Variations in the genes encoding nAChR subunits, DA receptors, or enzymes involved in nicotine metabolism may be responsible for

the reported differences between Lewis and Fischer 344 rats, but additional research is needed to test this hypothesis.

Comparisons between outbred rat strains, such as Sprague-Dawley and Long-Evans rats, have demonstrated that naturally occurring genetic variability can alter sensitivity to nicotine in several behavioral procedures. Both Sprague-Dawley and Long-Evans rats self-administered nicotine in a limited access procedure whereas Lewis and Fischer 344 rats did not, suggesting that the former two strains may be more sensitive to the rewarding properties of nicotine than Lewis and Fischer 344 inbred rats [151]. Additionally, Faraday and colleagues [43] reported that nicotine has differential effects on the acoustic startle response (ASR) and sensory gating, as measured by prepulse inhibition of the acoustic startle response (PPI). Specifically, nicotine enhanced ASR and PPI in Sprague-Dawley rats, but disrupted ASR and PPI in Long-Evans rats. The effects of chronic nicotine have also been characterized in these two strains: Long-Evans rats exhibited greater locomotor activity during chronic nicotine administration than Sprague-Dawley rats [44]. Thus, research comparing Sprague-Dawley and Long-Evans rats has revealed that genetic variability can alter sensitivity to nicotine, but one limitation with using outbred rat strains is that the greater genetic variability within an outbred strain will make it difficult to identify the variants of genes that modulate nicotine sensitivity.

Additional support for genetic influences on the effects of nicotine comes from comparisons of multiple strains of inbred mice, and subsequent research has demonstrated that polymorphisms in nAChR subunits may alter sensitivity to nicotine. A comparison of 19 inbred mouse strains revealed considerable differences in the effects of nicotine on respiration, heart rate, body temperature, acoustic startle response, and locomotor activity on the Y-maze [22, 104], and strain-dependent differences in nicotine and α -bungarotoxin binding sites were also reported [105]. Furthermore, inbred mouse strains developed tolerance to the effects of nicotine on these measures during chronic nicotine infusion, but the nicotine dose that produced tolerance varied between the inbred mouse strains that were tested [22, 106]. For instance, C57BL/6J mice developed tolerance to the physiological effects of nicotine when low doses of chronic nicotine were infused, whereas high doses were required to produce tolerance in C3H/2J mice [106]. Furthermore, inbred mouse strains that were more sensitive to low doses of acute nicotine were more likely to develop tolerance during chronic nicotine infusion [23, 106], suggesting that the gene variants that increase sensitivity to acute nicotine may also regulate the development of tolerance.

The effects of nicotine have been well characterized in the C57BL/6J inbred mouse strain; thus, several studies have compared the behavioral effects of nicotine and nicotine withdrawal in this strain to other inbred strains, such as DBA/2J mice³. C57BL/6J mice exhibited greater oral nicotine self-administration than DBA/2J, ST/J, BUB/J, A/J, and C3H/2J mice [140]. Furthermore, C57BL/6J mice developed nicotine CPP, whereas DBA/2J mice did not [60]. These data suggest that C57BL/6J mice have increased sensitivity to the rewarding properties of nicotine relative to other strains. Inbred mouse strains such as A/J and 129/SvEv mice were more sensitive to the antinociceptive effects of acute nicotine than C57BL/6J and DBA/2J mice [29]. However, C57BL/6J mice showed increased sensitivity to pain during nicotine-precipitated withdrawal, but precipitated withdrawal had no effect on pain perception in 129/SvEv mice [28]. Furthermore, nicotine-precipitated withdrawal also increased anxiety (as measured by decreased time in the open arms of the elevated plus maze) and somatic symptoms (e.g. increased head shakes, paw tremors, or scratching) in C57BL/6J mice but not 129/SvEv mice, suggesting that genetic factors mediate the severity of nicotine withdrawal symptoms [28]. Together, these data provide evidence that genetic variability can influence

³Substrains of an inbred mouse strain that are bred by separate sources (e.g. C57BL/6J vs. C57BL/6J) can exhibit differences in behavior due to genetic drift [158]. Therefore, the inbred mouse strains described here will make a distinction between breeders.

the effects of nicotine on reward and pain perception, as well as the symptoms observed during nicotine withdrawal. The C57BL/6 mouse strain may be more sensitive to many of the effects of nicotine relative to other strains, but it is important to note that this trend was not observed in all of the behavioral procedures tested.

One limitation of inbred strain studies is that it is difficult to determine the genes (and the variations of these genes) that are mediating the effects. However, inbred strains can be used to identify quantitative trait loci (QTL) associated with nicotine-related behaviors. These studies have identified loci containing nAChR subunit genes, and have found some regions associated with nicotine phenotypes that have also been linked to nicotine dependence in humans. Gill and colleagues [52] examined the effects of nicotine on locomotor activity in recombinant congenic strains (RCS) of mice. Briefly, RCS mice were generated by backcrossing F1 hybrid mice (C57BL/6J $\times$ A/J) with one of the progenitor strains (C57BL/6J or A/J mice), such that some RCS mice were backcrossed to A/J mice (AcB) whereas others were backcrossed to C57BL/6J mice (BcA). The offspring from the first backcross were also backcrossed to one of the two background strains; thus, the subsequent generation possessed ~12.5% of the genes from the donor strain and ~87.5% of the background strain. Following 20 or more generations of brother/sister inbreeding, the chromosomal maps from AcB/BcA RCS mice can be used to identify loci associated with nicotine-related phenotypes. In AcB mice, a single locus association analysis revealed QTL for the locomotor-enhancing effects of nicotine on chromosomes 11–14 and 17, and a locus on chromosome 11 was linked to the locomotor-suppressant effects of nicotine. Furthermore, seven loci located on chromosomes 2, 7, 8, 13, 14, 16 and 17 were linked to nicotine-induced increases in locomotion in BcA mice [52]. Interestingly, the locus on chromosome 14 associated with the effects of nicotine on locomotion in BcA mice contains the *Chrna2* gene, suggesting that nAChR subunit genes may play an important role in sensitivity to the effects of nicotine on locomotion [52]. Additional research with inbred mice has also identified loci associated with oral nicotine consumption. Previous research has demonstrated substantial differences in oral nicotine consumption between C57BL/6J and C3H/HeJ mice [140]; thus, Li and colleagues [97] used a genetic cross between these two strains to identify loci associated with sensitivity to oral nicotine consumption. Four loci associated with oral nicotine consumption (located on chromosomes 1, 4, 7, and 15) were identified in F2 hybrid mice [97]. The locus found on chromosome 1 is syntenic with the human chromosome 1, and this region has also been associated with smoking quantity in humans [182]. Together, these data suggest that nAChR subunit genes play an important role in mediating sensitivity to nicotine, and reveal that some loci associated with nicotine phenotypes may be common across multiple species.

Although research from inbred rodent strains has demonstrated that genetic variability can alter sensitivity to nicotine, many of the specific genes that regulate nicotine sensitivity remain unknown. However, polymorphisms in the *Chrna4*, *Chrna5*, and *Chrna7* genes have been identified that may influence the effects of nicotine in inbred mouse strains. Stitzel and colleagues [159] identified restriction fragment length polymorphisms (RFLPs) between C3H/2Ibg and DBA/2Ibg mice in both *Chrna5* and *Chrna7*, and used a classic genetic cross between these two strains to determine whether these RFLPs are associated with sensitivity to nicotine-induced seizures. F2 hybrid mice that were homozygous for the C3H/2Ibg variant of the *Chrna5* RFLP had greater vulnerability for nicotine-induced seizures than mice that were homozygous for the DBA/2Ibg variant of this RFLP. Furthermore, the C3H/2Ibg variant of the *Chrna7* RFLP increased susceptibility for nicotine-induced seizures and mice that possessed C3H/2Ibg alleles for both *Chrna5* and *Chrna7* had greater sensitivity to nicotine-induced seizures than mice carrying only one of the C3H/2Ibg alleles, suggesting that the two RFLPs function in an additive manner to increase sensitivity [159].

A SNP in *Chrna4* that results in an alanine or threonine amino acid substitution (A529T) at position 529 of the $\alpha 4$ nAChR subunit has also been linked to strain-dependent differences in responses to nicotine [160]. This SNP was associated with changes in the functional properties of $\alpha 4$ containing receptors: nicotine-induced $^{86}\text{Rb}^+$ efflux was significantly greater in mice that possess the A529 variant [36]. Additionally, mice carrying the A529 variant of this SNP were less sensitive to the effects of nicotine on locomotor activity (measured on the Y-maze), body temperature, and respiration when compared to mice that have the T529 variant [172, 173]. However, A529 mice were more sensitive to nicotine-induced changes in acoustic startle relative to T529 mice [173]. Furthermore, a comparison of 14 inbred mouse strains revealed that mice carrying the A529 variant of this SNP consumed less nicotine than mice with the T529 variant [13]. Furthermore, Butt and colleagues studied nicotine preference and consumption in F2 mice generated by crossing A/J mice with C57BL/6J mice that lack the $\beta 2$ nAChR subunit. F2 mice that possessed the A529 variant showed significantly less preference and consumption of nicotine, but only if they were $\beta 2$ subunit WT mice [13]. The fact that the association between A529T and nicotine preference and consumption was not present in $\beta 2$ KO mice suggests that variability in either *Chrna4* or *Chrn2* is mediating nicotine preference and consumption. Together, these data suggest that variability in *Chrna4* can influence sensitivity to nicotine, and demonstrate that the use of both inbred mouse strains and genetic KO mice can provide new insight into the genes that are responsible for individual differences in the response to nicotine.

Overall, research comparing the effects of nicotine in multiple inbred rodent strains has provided strong evidence that naturally occurring genetic variability can modulate the response to nicotine. Furthermore, research by Stitzel and colleagues has demonstrated that variability in nAChR subunit genes is associated with changes in the functional properties of receptors and in some of the behavioral effects of nicotine. These data correspond with research in humans that has linked variability in nAChR subunit genes to smoking phenotypes, suggesting that additional research in mice may lead to a better understanding of how individual differences in genetics can increase or decrease vulnerability to nicotine addiction. Future research should investigate whether variability in other nAChR subunit genes are associated with changes in nicotine sensitivity.

The Effects of Nicotine in Genetic Knockout and Knockin Mice

The use of genetically manipulated animals that have had a gene of interest inserted or deleted (KI and KO mice, respectively) has allowed researchers to uncover details about the possible functions of nAChRs and also has revealed that the genes encoding nAChRs mediate variability in many of the behavioral effects of nicotine. To date, knockout mice have been generated that lack the $\alpha 3$ [191], $\alpha 4$ [108], $\alpha 5$ [183], $\alpha 6$ [19], $\alpha 7$ [123], $\alpha 9$ [177], $\beta 2$ [129,190], $\beta 3$ [27], and $\beta 4$ [190] nAChR subunits, and subsequent research has demonstrated that the deletion of these genes will alter many of the effects of nicotine in mice. Additionally, KI mice with point mutations that produce hypersensitive nAChRs have been generated for the $\alpha 4$ [86,167] and $\alpha 7$ subunits [124]. Although genetically modified mice can help to determine the possible functions of a given gene, there are some limitations to using this approach [20,162]. For instance, developmental compensatory mechanisms that result from the deletion of a gene could alter the function of other nAChRs or other processes. In addition, nAChR subunit KO mice generated using 129/SvEv mice that are backcrossed onto a C57BL/6J background may have genes flanking the targeted nAChR subunit gene that carry 129/SvEv alleles, rather than the alleles found in C57BL/6J mice [187]. Therefore, it is possible that differences in flanking genes may be responsible for the effects of nicotine observed in KO mice. Another potential complication with the phenotypes observed in genetic KO mice is that some sequences within the targeting vector, such as the promoter region of a selectable marker, may alter the expression of genes located near the gene targeted for deletion [121]. Finally, the deletion of some genes

can produce a dramatically shortened lifespan (e.g. $\alpha 3$ KO mice) or result in embryonic death. However, the development of conditional KO mice, which have a target gene deleted only in a given brain region, can address this issue.

The $\beta 2$ nAChR Subunit

The $\beta 2$ subunit was the first nAChR subunit successfully targeted for deletion [129], and research with KO mice has demonstrated that $\beta 2$ -containing nAChRs are required for many of the effects of nicotine, including alterations of reward, avoidance learning, and hippocampus-dependent learning. In addition, $\beta 2$ -containing nAChRs also mediate several nicotine withdrawal symptoms (see Table 2 for a summary of nAChR subunit KO studies). $\beta 2$ subunit KO mice do not self-administer nicotine and do not show nicotine-evoked increases in DA release in the ventral striatum, in contrast to WT littermates [7,130]. Similarly, $\beta 2$ KO mice do not exhibit nicotine CPP, whereas wild-type (WT) mice can learn to associate a previously neutral context with nicotine administration [181]. Additional research has also demonstrated that intra-VTA nicotine self-administration can be restored in $\beta 2$ KO mice by administering a lentiviral vector into the VTA that expresses the $\beta 2$ subunit [110], which suggests that $\beta 2$ -containing nAChRs in the VTA are sufficient for the rewarding effects of nicotine. Together, these studies provide strong evidence that the rewarding properties of nicotine require $\beta 2$ -containing nAChRs, but it remains unknown whether naturally occurring genetic variability in *Chrn2* will alter sensitivity to the rewarding effects of nicotine.

In addition to mediating nicotine-induced changes in reward, $\beta 2$ -containing nAChRs are also critically involved in the effects of nicotine on avoidance learning. Avoidance learning has been assessed using several behavioral procedures, including passive avoidance, conditioned place aversion (CPA), and conditioned taste aversion (CTA). In the passive avoidance procedure, mice are placed in a well-lit portion of a chamber and receive a footshock when they cross over to the dark portion of the chamber. An association between the dark chamber and the footshock is assessed on testing day by measuring the time to enter the dark chamber [157]. Research with $\beta 2$ KO mice has demonstrated that saline-treated KO mice exhibit greater passive avoidance relative to WT mice; however, nicotine enhances passive avoidance in WT mice but has no effect in KO mice [129]. In the nicotine CPA procedure, mice treated with chronic nicotine learn to avoid a chamber that is paired with nAChR antagonist-precipitated withdrawal [163]. $\beta 2$ KO mice do not show nicotine CPA whereas WT mice do [77]. Finally, mice trained in the nicotine CTA procedure learn to avoid flavored water that is paired with nicotine [53]; a study investigating CTA in $\beta 2$ KO mice has shown that KO mice have reduced CTA relative to WT mice [152]. Overall, research using several behavioral procedures has consistently demonstrated that the effects of nicotine on avoidance learning, which models the effects of nicotine on affect and cognition in humans, are mediated by $\beta 2$ -containing nAChRs.

Acute nicotine enhances hippocampus-dependent forms of learning, such as trace cued conditioning [56,54] and contextual conditioning [34,57], whereas withdrawal from chronic nicotine produces deficits in contextual conditioning [33,30,132,135]. Research with KO mice has demonstrated that the effects of nicotine on hippocampus-dependent forms of learning require the $\beta 2$ nAChR subunit. In trace cued conditioning, an auditory CS is separated by an interval of time before mice receive a footshock US. Mice acquire a context-US and a CS-US association, but the insertion of a time interval between the CS and US requires working memory to be engaged [15]. Therefore, the hippocampus is required for both the context-US and CS-US associations [114,134]. Research from our lab has demonstrated that $\beta 2$ KO mice show no enhancement of trace cued conditioning by acute nicotine, whereas WT littermates do [34]. Similarly, acute nicotine enhances contextual conditioning in $\beta 2$ WT mice but not in KO mice, suggesting that the effects of acute nicotine on hippocampus-dependent learning likely require $\alpha 4\beta 2^*$ nAChRs [186]. Furthermore, nicotine withdrawal-related deficits in

contextual conditioning are present in $\beta 2$ subunit WT mice but not in KO mice [133]. Taken together, these data reveal that $\beta 2$ -containing nAChRs are required for the enhancing effects of nicotine on hippocampus-dependent learning, as well as for the disruption of learning that occurs during nicotine withdrawal. It remains unknown whether variability in the mouse *Chrn2* gene can influence sensitivity to the effects of acute nicotine and nicotine withdrawal on hippocampus-dependent learning.

Symptoms of nicotine withdrawal have been studied using $\beta 2$ KO mice, including withdrawal-related changes in anxiety, hippocampus-dependent learning (described in the previous paragraph), and somatic symptoms of withdrawal. $\beta 2$ subunit KO mice withdrawn from chronic nicotine do not exhibit withdrawal-associated increases in anxiety, as measured by the elevated plus maze [77]. In contrast, the somatic symptoms of nicotine withdrawal are present in both $\beta 2$ KO and WT mice, suggesting that the somatic symptoms of nicotine withdrawal are mediated by other nAChR subunits [7,77,146]. Research with other nAChR subunit KO mice has demonstrated that the $\beta 4$ [146], $\alpha 5$ [77], and $\alpha 7$ nAChR subunits [148] likely mediate the somatic symptoms of withdrawal. Thus, these studies provide evidence that the various symptoms of nicotine withdrawal are mediated by different nAChR subunits.

Overall, research from genetically modified mice has provided strong evidence that $\beta 2$ -containing nAChRs are required for the effects of nicotine on reward, avoidance learning, hippocampus-dependent learning, and withdrawal-related changes to learning and anxiety. Although these data are consistent with research in humans that have implicated the *CHRNA2* gene in some of effects of nicotine, the data collected in humans on the role of *CHRNA2* in nicotine addiction has been mixed. One possible explanation for this discrepancy between human and animal research is that all of the phenotypes studied in $\beta 2$ KO and WT mice have yet to be tested in humans, and therefore it is possible that variability in *CHRNA2* in humans may be associated with the effects of nicotine on reward, cognition, and withdrawal-induced alterations of learning and anxiety. Additionally, research in humans has found associations between *CHRNA2* and smoking phenotypes [40]; therefore future research could address whether polymorphisms in the mouse *Chrn2* gene may also modulate the effects of nicotine.

The $\alpha 4$ nAChR Subunit

Mice lacking the $\alpha 4$ subunit exhibit several similarities to $\beta 2$ KO mice, providing further evidence that $\alpha 4\beta 2^*$ nAChRs likely mediate many of the effects of nicotine. For instance, both $\alpha 4$ and $\beta 2$ KO mice do not have high affinity binding sites for nicotine [108,129], exhibited a reduced antinociceptive effect in a hot-plate test [108], and showed reduced sensitivity to the effects of nicotine on locomotor activity and nicotine-induced hypothermia [113,168,174]. Furthermore, $\alpha 4$ and $\beta 2$ KO mice do not show nicotine-induced increases in striatal DA, whereas their respective WT littermates do [109,130]. However, basal striatal DA levels were significantly greater in $\alpha 4$ KO mice than in WT mice [109]. An additional abnormality in dopaminergic function has been reported: $\alpha 4$ KO mice have reduced DA transporter function when compared to WT mice [126]. Although the similarities between $\beta 2$ and $\alpha 4$ KO mice in response to nicotine suggest that $\alpha 4\beta 2^*$ nAChRs are involved in the effects of nicotine on pain perception, locomotor activity, body temperature, and nicotine-induced changes in DA release, many of the phenotypes examined in $\beta 2$ KO mice have yet to be tested in $\alpha 4$ subunit KO mice. Given that $\beta 2$ subunits can interact with several subunits other than $\alpha 4$ to form receptors [119], it is possible that some of the effects observed in $\beta 2$ KO mice are attributable to associations with non- $\alpha 4$ subunits (e.g. $\alpha 2$, $\alpha 3$, $\alpha 5$, or $\alpha 6$). Thus, a more complete assessment of the effects of nicotine in $\alpha 4$ subunit KO mice is necessary.

In addition to $\alpha 4$ subunit KO mice, two lines of $\alpha 4$ KI mice have been generated that have either a leucine to serine (Leu9'Ser) or a leucine to alanine (Leu9'Ala) point mutation in the pore-forming M2 transmembrane domain of the $\alpha 4$ subunit [86,167]. In Leu9'Ser mice, this point mutation produces $\alpha 4$ -containing nAChRs with a ~30-fold increase in sensitivity to acetylcholine and nicotine. The mutation is lethal to homozygous mice but not to heterozygous mice that possess a neomycin resistance cassette [86]. Subsequent research has demonstrated that Leu9'Ser heterozygous mice are more sensitive to the antinociceptive effects of nicotine than WT mice [29]. Although *in vivo* research using Leu9'Ser mice is limited to heterozygotes, Tapper and colleagues [167] have shown that Leu9'Ala homozygous mice are viable and will exhibit a ~50-fold increase in sensitivity to nicotine, develop nicotine CPP at a ~50-fold lower dose of nicotine than in WT mice, and develop tolerance to nicotine-induced changes in body temperature at a ~50-fold lower dose of nicotine. Furthermore, a recent study that utilized both $\alpha 4$ KO and Leu9'Ala KI mice demonstrated that if the responses to nicotine-induced changes to body temperature and locomotor activity in both mutant lines were summed together, this predicted response would resemble the effects of nicotine in WT mice [168]. These data suggest that $\alpha 4$ -containing nAChRs are both necessary and sufficient for the effects of nicotine on body temperature and locomotor activity. Research utilizing $\alpha 4$ KI mice reveals that genetic variability in the $\alpha 4$ subunit can produce dramatic changes to nicotine sensitivity, and suggest that naturally occurring variability in the human *CHRNA4* gene may alter sensitivity to nicotine and increase vulnerability to nicotine addiction. Additionally, the existence of both a KO and hypersensitive KI strain can allow future research to better characterize the contributions of $\alpha 4$ and non- $\alpha 4$ containing nAChRs to the effects of nicotine.

The $\alpha 7$ nAChR Subunit

The homomeric $\alpha 7$ nAChR is abundantly expressed throughout the brain [68,90], and likely plays an important role in the effects of nicotine. However, research using $\alpha 7$ subunit KO mice has found few differences in the effects of nicotine between KO and WT mice. Research examining the rewarding properties of nicotine has found that both $\alpha 7$ KO and WT mice can develop nicotine CPP, suggesting that $\alpha 7$ nAChRs are not critical for the effects of nicotine on reward [181]. The enhancing effects of acute nicotine on contextual conditioning and trace cued conditioning are also normal in $\alpha 7$ KO mice, but only when a lower footshock US intensity is used [34,186]. At higher footshock intensities, both $\alpha 7$ KO and WT mice do not show enhancement of contextual conditioning by acute nicotine [186]. In addition, Jackson and colleagues [77] have found no difference between $\alpha 7$ KO and WT mice in avoidance learning as measured by nicotine CPA. Finally, the locomotor suppressant effect of acute nicotine and tolerance to the effects of nicotine on locomotion are equivalent between $\alpha 7$ KO and WT mice [122]. Although these data suggest that $\alpha 7$ nAChRs are not critically involved in many of the effects of nicotine, these results do not rule out the possibility that $\alpha 7$ nAChRs play an ancillary role in these phenotypes. Furthermore, it is possible that compensatory mechanisms in $\alpha 7$ KO mice may obscure the role of this subunit in the effects of nicotine. In support, $\alpha 7$ KO mice have enhanced trace cued conditioning at higher footshock intensities, regardless of whether nicotine or saline was administered [34].

Investigations of nicotine withdrawal symptoms have demonstrated that $\alpha 7$ nAChRs play a role in withdrawal-related changes in somatic symptoms and hyperalgesia [59,77,148], but do not play a crucial role in other withdrawal symptoms. Salas and colleagues [148] reported that somatic signs during nAChR antagonist-precipitated withdrawal are reduced in $\alpha 7$ KO mice relative to WT littermates. In addition, studies using two different methods of chronic nicotine administration (either chronic oral nicotine or osmotic mini-pumps) have demonstrated that nicotine withdrawal-induced hyperalgesia is present in $\alpha 7$ WT mice, but not in KO mice [59, 77]. Although these data suggest that $\alpha 7$ nAChRs mediate some aspects of nicotine withdrawal, other research has found that withdrawal-associated changes in contextual learning and anxiety

are not affected by the deletion of the $\alpha 7$ subunit. Specifically, $\alpha 7$ KO and WT mice both exhibit nicotine withdrawal-related deficits in contextual conditioning [133]. Furthermore, withdrawal-associated increases in anxiety, as measured by decreased time in the open arms of the elevated plus maze, were present in both KO and WT mice [77]. Thus, $\alpha 7$ nAChRs are involved in select nicotine withdrawal symptoms.

Research with $\alpha 7$ KI mice suggest that alterations in the functional properties of $\alpha 7$ nAChRs can lead to significant changes in sensitivity to nicotine. Specifically, homozygous $\alpha 7$ KI mice that have a leucine to threonine (L250T) point mutation in the pore-forming domain of the $\alpha 7$ subunit will die after birth, but heterozygotes remain viable and exhibit increased sensitivity to nicotine induced seizures [11,51,124]. These studies reveal that a gain of function in the $\alpha 7$ nAChR can produce substantial alterations of nicotine-related phenotypes; thus, a more complete evaluation of the effects of nicotine in $\alpha 7$ KI mice is necessary. Furthermore, research with $\alpha 7$ KI mice suggest that naturally occurring variants of *CHRNA7* in humans may enhance sensitivity to nicotine and increase vulnerability to nicotine addiction; future candidate gene association studies could evaluate associations between *CHRNA7* and smoking phenotypes.

Additional nAChR Subunits

Similar to humans, the genes that encode the $\alpha 6$ and $\beta 3$ nAChR subunits exist in a cluster located on chromosome 8; $\alpha 6$ and $\beta 3$ KO mice have been generated, but few phenotypes have been evaluated in these mice. Nicotine stimulates DA release in reward-related brain areas such as the striatum, and this effect is partially inhibited by α -conotoxin MII (α CtxMII) [85]. Research with $\alpha 6$ and $\beta 3$ KO mice has demonstrated α CtxMII inhibits nAChRs that contain both of these subunits. Champiaux and colleagues [19] reported no gross anatomical abnormalities in $\alpha 6$ KO mice, but $\alpha 6$ KO mice no longer possess high affinity binding sites for [125 I] α CtxMII. In $\beta 3$ KO mice, the inhibition of nicotine-induced DA release by α CtxMII is virtually absent [27,149]. $\beta 3$ KO mice also exhibit greater activity in an open field arena, have decreased prepulse inhibition of the acoustic startle response, and show reduced anxiety relative to WT mice [9,27]. These data suggest that $\beta 3$ -containing nAChRs may play a role in anxiety, but the effects of nicotine on these behavioral procedures remain unknown. Furthermore, given that research in humans has implicated the *CHRNA6* and *CHRNA3* genes in modulating nicotine dependence, testing of the effects of nicotine in $\alpha 6$ and $\beta 3$ KO mice may clarify the role of this gene cluster in nicotine dependence-related behaviors.

The genes encoding the $\alpha 5$, $\alpha 3$, and $\beta 4$ subunits have been successfully targeted for deletion in mice, but many of the effects of nicotine have yet to be studied in these KO mice. $\alpha 5$ KO mice do not appear to have any physical abnormalities and have normal mRNA expression of non- $\alpha 5$ nAChR subunits, but $\alpha 5$ KO mice have substantially reduced sensitivity to nicotine-induced seizures and are resistant to the locomotor suppressant effects of nicotine [144]. Furthermore, $\alpha 5$ KO mice have reduced somatic signs during nAChR antagonist-precipitated withdrawal, but show normal precipitated withdrawal-related changes in pain perception and anxiety relative to WT mice; nicotine CPA in $\alpha 5$ KO mice is also similar to WT mice [77]. $\alpha 3$ KO mice have several physical abnormalities and die within 1–8 weeks after birth, but heterozygous mice do not have any gross physical deformities and can be used to study the behavioral effects of nicotine [191]. Salas and colleagues [147] have demonstrated that $\alpha 3$ heterozygous mice are less sensitive to nicotine-induced seizures but showed normal suppression of locomotor activity in response to acute nicotine. Finally, $\beta 4$ KO mice have been tested for several phenotypes and show some similarity to $\alpha 3$ and $\alpha 5$ KO mice. For example, $\beta 4$ KO mice exhibit reduced sensitivity to the hypolocomotor effects of nicotine, have increased resistance to nicotine-induced seizures, and show reduced somatic signs during nAChR antagonist-precipitated withdrawal when compared to WT mice [147,146]. Furthermore, the core body temperature of $\beta 4$ KO mice is lower than WT mice, and the effects of nicotine on

body temperature are reduced in the KO mice [143]. Additionally, Salas and colleagues [145] evaluated anxiety in $\beta 4$ KO and WT mice; $\beta 4$ KO mice exhibited less changes in heart rate in the open arms of the elevated plus maze when compared with WT mice, but no differences were reported in other measures of anxiety. Thus, research with $\alpha 5$, $\alpha 3$, and $\beta 4$ KO mice has revealed that related genes may mediate similar effects of nicotine: all three lines of genetically modified mice exhibit resistance to nicotine-induced seizures, whereas the $\alpha 5$ and $\beta 4$ subunits mediate both the locomotor suppressant effects of nicotine and somatic signs of nicotine withdrawal. Given that the genes that encode the $\alpha 5$, $\alpha 3$, and $\beta 4$ subunits exist as a cluster in both mice (chromosome 9) and humans (chromosome 15), data suggest that this gene cluster may regulate multiple effects of nicotine.

In summary, research using genetically modified mice has demonstrated that nAChR subunits mediate the effects of nicotine on reward, associative learning, locomotor activity, pain perception, and several symptoms of nicotine withdrawal. These data in mice are consistent with research in humans, and suggest that data obtained from genetic KO/KI studies may help to determine the functions of these genes in humans. For instance, the effects of nicotine are similarly altered in $\alpha 5$, $\alpha 3$, and $\beta 4$ KO mice; thus, multiple genes in the *CHRNA5-CHRNA3-CHRNA4* gene cluster in humans may be critically involved in the behavioral effects of nicotine. It is important to note that one major limitation of research with genetically manipulated mice is that the deletion of a given nAChR subunit does not reveal whether variability in this gene can alter the response to nicotine. However, this issue can be addressed by observing the effects of nicotine in both KO and KI mice, as was done recently by Tapper and colleagues [168]. Additionally, Butt and colleagues [13] have demonstrated that stronger links between genetic variability and sensitivity to nicotine can be established by crossing inbred mouse strains with KO mice. Thus, future research examining the genetic factors that influence nicotine addiction should utilize these more powerful research designs.

Conclusion

Nicotine addiction is major worldwide health problem, and a more complete understanding of how genetic variability can influence vulnerability to addiction can explain individual differences in response to nicotine and in response to smoking cessation treatments. Additionally, a better understanding of genetic influences on nicotine addiction can aid in the development of more effective therapeutics. The reviewed research in humans has provided evidence that variability in nAChR subunit genes is associated with multiple smoking phenotypes, and that these effects depend upon other factors such as gender or ethnicity. These findings are consistent with studies in inbred strains of rats and mice, which exhibit significant strain-dependent differences in the response to nicotine. Furthermore, research with genetically manipulated mice has shown that nAChR subunit genes mediate the effects of nicotine, and that artificial changes in the amino acid sequence of these proteins can produce substantial changes in the functional properties of these receptors and will also alter the behavioral effects of nicotine. Naturally occurring variability in nAChR subunit genes in mice has also been linked to changes in receptor function and in the behavioral effects of nicotine. Although much remains unknown regarding the role of nAChR subunit genes in nicotine addiction, an important next step in understanding the genetic factors that modulate nicotine addiction will be the combined use of inbred mouse strains and KO mice to link genetic variability in nAChR subunit genes with background genotype.

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Table 1

Summary of Candidate Gene Association Studies

Gene/SNP ID	Sample	Alleles	Location	Phenotypes	References
CHRNA3					
rs578776	Australians/Americans (European Ancestry)	G/A	3'UTR	FTND	Saccone et al., 2007
rs1051730	Australians/Americans (European Ancestry)	G/A	Exon (Syn)	FTND	Saccone et al., 2007
rs3743078	Australians/Americans (European Ancestry)	G/C	Intron	FTND	Saccone et al., 2007
rs6495308	European Ancestry	C/T	Intron	SQ	Berrettini et al., 2008
rs1317286	European Ancestry	G/A	Intron	SQ	Berrettini et al., 2008
rs1051730	European Ancestry	G/A	Exon (Syn)	Lung Cancer	Amos et al., 2008
rs1051730	European Ancestry	G/A	Exon (Syn)	SQ	Thorgeirsson et al., 2008
rs1051730	European Ancestry	G/A	Exon (Syn)	FTND, DSM-IV Nicotine Dependence	Thorgeirsson et al., 2008
rs1051730	European Ancestry	G/A	Exon (Syn)	Lung Cancer	Thorgeirsson et al., 2008
rs1051730	Diverse	G/A	Exon (Syn)	Peripheral Arterial Disease	Thorgeirsson et al., 2008
rs1051730	Diverse	G/A	Exon (Syn)	Lung Cancer	Hung et al., 2008
CHRNA4					
rs1044396	Chinese Men	C/T	Exon (Syn)	FTND, RTQ, Nicotine Addiction	Feng et al., 2004
rs1044397	Chinese Men	G/A	Exon (Syn)	FTND, RTQ, Nicotine Addiction	Feng et al., 2004
rs1044396	European Americans	C/T	Exon (Syn)	SQ	Li et al., 2005
rs3787137	European American Men	G/A	Intron	SQ	Li et al., 2005
rs2236196	African American Women	G/A	3' UTR	SQ, HSI, FTND	Li et al., 2005
rs2273504	African and European American Women	G/A	Intron	FTND	Li et al., 2005
rs2236196	Diverse (80% Caucasian)	C/T	3' UTR	Subjective Responses, Smoking Cessation	Hutchison et al., 2007
rs6122429	Diverse (80% Caucasian)	G/A	Promotor Region	Subjective Responses	Hutchison et al., 2007
CHRNA5					
rs16969968	Australians/Americans (European Ancestry)	G/A	Exon (NSyn)	FTND	Saccone et al., 2007
rs637137	Australians/Americans (European Ancestry)	A/T	Intron	FTND	Saccone et al., 2007
rs684513	Australians/Americans (European Ancestry)	G/C	Intron	FTND	Saccone et al., 2007
rs514743	Diverse (72% Caucasian)	A/T	Intron	Smoking Initiation	Schlaepfer et al., 2007
rs16969968	Diverse	G/A	Exon (NSyn)	Lung Cancer	Hung et al., 2008
CHRNA6					
rs2304297	Australians/Americans (European Ancestry)	G/C	3'UTR	FTND	Saccone et al., 2007
rs2304297	Diverse (74% Caucasian)	G/C	3'UTR	Subjective Responses	Zeiger et al., 2007
CHRNA2					
rs2072660	Israeli Women	C/T	3'UTR	Smoking Initiation	Greenbaum et al., 2006
rs2072658	Diverse (72% Caucasian)	G/A	Near Gene	Subjective Responses	Ehringer et al., 2007
rs2072660	Diverse (72% Caucasian)	C/T	3'UTR	Subjective Responses	Ehringer et al., 2007
CHRNA3					
rs13277254	Australians/Americans (European Ancestry)	G/A	Intron	FTND	Bierut et al., 2007
rs6474413	Australians/Americans (European Ancestry)	C/T	Promotor Region	FTND	Bierut et al., 2007
rs10958726	Australians/Americans (European Ancestry)	G/T	Promotor Region	FTND	Saccone et al., 2007
rs6474413	Australians/Americans (European Ancestry)	C/T	Promotor Region	FTND	Saccone et al., 2007
rs4953	Australians/Americans (European Ancestry)	G/C	Exon (Syn)	FTND	Saccone et al., 2007
rs4952	Australians/Americans (European Ancestry)	C/T	Exon (Syn)	FTND	Saccone et al., 2007

Gene/SNP ID	Sample	Alleles	Location	Phenotypes	References
rs4950	Diverse (74% Caucasian)	G/A	Exon (Syn)	Subjective Responses	Zeiger et al., 2007
rs13280604	Diverse (74% Caucasian)	G/A	Intron	Subjective Responses	Zeiger et al., 2007
hCV25772398	Diverse (74% Caucasian)	C/T	Intron	Subjective Responses	Zeiger et al., 2007
rs4953	Diverse (74% Caucasian)	G/C	Exon (Syn)	Subjective Responses	Zeiger et al., 2007
CHRNA4					
rs3813567	Australians/Americans (European Ancestry)	G/A	Near Gene	FTND	Saccone et al., 2007
rs1948	Diverse (72% Caucasian)	C/T	3' UTR	Smoking Initiation	Schlaepfer et al., 2007
rs8023462	Diverse (72% Caucasian)	C/T	Intergenic	Smoking Initiation	Schlaepfer et al., 2007

Legend: A = Adenine; C = Cytosine; G = Guanine; T = Thymine; Syn = Synonymous SNP; NSyn = Non-synonymous SNP; UTR = Untranslated Region FTND = Fagerström test for nicotine dependence; RTQ = Revised Tolerance Questionnaire; SQ = Smoking Quantity; HSI = Heaviness of Smoking Index

Table 2
The Effects of Nicotine in nAChR Knockout and KnockIn Mice

Gene	Effect in KO/KI Mice	References
<i>CHRNA3</i>		
KO Mice	+/- mice less sensitive to nicotine-induced seizures Effects of nicotine on locomotion are normal	Salas et al., 2004b Salas et al., 2004b
<i>CHRNA4</i>		
KO Mice	No high-affinity binding sites for nicotine Reduced antinociception (Hot-Plate Test) No nicotine-induced changes in body temperature No nicotine-induced changes in locomotion Nicotine-induced increases in DA not present Reduced DA transport functioning	Marubio et al., 1999 Marubio et al., 1999 Tapper et al., 2007 Tapper et al., 2007 Marubio et al., 2003 Parish et al., 2005
Leu9'Ser KI mice	KI mice have a 30-fold increase in nicotine/acetylcholine sensitivity Nicotine-induced changes in pain perception are enhanced in +/- mice	Labarca et al., 2001 Damaj et al., 2007b
Leu9'Ala KI mice	KI mice have a 50-fold increase in nicotine sensitivity Develop nicotine CPP at 50-fold lower dose than WT Tolerance to nicotine can occur with a 50-fold lower dose than WT Nicotine-induced changes in body temperature are enhanced Nicotine-induced changes in locomotion are enhanced	Tapper et al., 2004 Tapper et al., 2004 Tapper et al., 2004 Tapper et al., 2007 Tapper et al., 2007
<i>CHRNA5</i>		
KO Mice	Reduced sensitivity to nicotine-induced seizures Effects of nicotine on locomotion are reduced Somaic signs of nicotine withdrawal are disrupted Normal nicotine withdrawal-induced hyperalgesia Normal nicotine withdrawal-induced increases in anxiety Normal nicotine CPA	Salas et al., 2003b Salas et al., 2003b Jackson et al., 2008 Jackson et al., 2008 Jackson et al., 2008 Jackson et al., 2008
<i>CHRNA6</i>		
KO Mice	No high affinity α CtxMII-sensitive binding sites	Champtiaux et al., 2002
<i>CHRNA7</i>		
KO Mice	Normal nicotine CPP Normal nicotine enhanced contextual conditioning Normal nicotine withdrawal-induced deficits in contextual conditioning Normal nicotine enhanced trace cued conditioning Normal nicotine CPA Normal nicotine withdrawal-related increases in anxiety Normal nicotine-induced changes in locomotion Normal tolerance to nicotine-induced changes in locomotion Somaic signs of nicotine withdrawal are disrupted Nicotine withdrawal-related hyperalgesia is reduced	Walters et al., 2006 Wehner et al., 2004 Davis and Gould, 2007b Portugal et al., 2008 Davis and Gould, 2007b Jackson et al., 2008 Jackson et al., 2008 Naylor et al., 2005 Naylor et al., 2005 Salas et al., 2007 Grabus et al., 2005
L250T KI Mice	Enhanced sensitivity to nicotine-induced seizures in +/- mice	Gil et al., 2002
<i>CHRNA2</i>		
KO Mice	No nicotine CPP No nicotine self-administration Nicotine-induced increases in DA not present No nicotine enhanced contextual conditioning No nicotine withdrawal-induced deficits in contextual conditioning No nicotine enhanced trace cued conditioning Enhanced PA, but nicotine does not alter PA in KO mice No nicotine CPA Reduced nicotine CTA No nicotine withdrawal-related increases in anxiety Somaic signs of nicotine withdrawal are normal No high-affinity binding sites for nicotine Reduced antinociception (Hot-Plate Test) No nicotine-induced changes in body temperature No nicotine-induced changes in locomotion	Walters et al., 2006 Picciotto et al., 1998 Besson et al., 2006 Picciotto et al., 1998 Wehner et al., 2004 Portugal et al., 2008 Davis and Gould, 2007b Picciotto et al., 1995 Jackson et al., 2008 Shoaib et al., 2002 Jackson et al., 2008 Salas et al., 2004a Besson et al., 2006 Jackson et al., 2008 Picciotto et al., 1995 Marubio et al., 1999 Tritto et al., 2004 McCallum et al., 2006 Tritto et al., 2004 McCallum et al., 2006

Gene	Effect in KO/KI Mice	References
Lentiviral re-expression	Re-expression of $\beta 2$ nAChRs in VTA restores self-administration	Maskos et al., 2005
<i>CHRNA3</i>		
KO Mice	No inhibition of nicotine-induced DA release by α CtxMII Enhanced open field locomotor activity Reduced PPI Decreased anxiety (elevated plus maze)	Cui et al., 2003 Salminen et al., 2004 Cui et al., 2003 Cui et al., 2003 Booker et al., 2007
<i>CHRNA4</i>		
KO Mice	Reduced sensitivity to nicotine-induced seizures Effects of nicotine on locomotion are reduced Somaic signs of nicotine withdrawal are disrupted No nicotine-induced changes in body temperature Decreased anxiety (elevated plus maze)	Salas et al., 2004b Salas et al., 2004b Salas et al., 2004a Sack et al., 2005 Salas et al., 2003a

Legend: KO = Knockout; KI = Knockin; WT = Wild-type; DA = Dopamine; α CtxMII = α Conotoxin MII; CPP = Conditioned Place Preference; CPA = Conditioned Place Aversion; CTA = Conditioned Taste Aversion; PA = Passive Avoidance; VTA = Ventral Tegmental Area; PPI = Prepulse Inhibition