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Natural Genetic Variability of the Neuronal Nicotinic Acetylcholine Receptor Subunit Genes in Mice: Consequences and Confounds

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Abstract

Recent human genetic studies have identified genetic variants in multiple nicotinic acetylcholine receptor (nAChR) subunit genes that are associated with risk for nicotine dependence and other smoking-related measures. Genetic variability also exists in the nAChR subunit genes in mice. Most studies on mouse nAChR subunit gene variability to date have focused on *Chrna4*, the gene that encodes the $\alpha 4$ nAChR subunit and *Chrna7*, the gene that encodes the $\alpha 7$ nAChR subunit. However, genetic variability exists for all nAChR genes in mice. In this review, we will describe what is known about nAChR subunit gene polymorphisms in mice and how it relates to variability in nAChR expression and function in brain. The relationship between nAChR genetic variability in mice and the effects of nicotine on several behavioral and physiological measures also will be discussed. In addition, an overview of the contribution of other genetic variation to nicotine sensitivity in mice will be provided. Finally, the potential for natural genetic variability to confound and/or modify the results of studies that utilize genetically engineered mice will be considered. As an example of the ability of a natural genetic variant to modify the effect of an engineered mutation, data will be presented that demonstrate that the effect of *Chrna5* deletion on oral nicotine intake is dependent upon naturally occurring variant alleles of *Chrna4*.

Keywords

nicotinic receptor; genetics; polymorphism; mice

1.1 Genetics of smoking in humans and nicotine sensitivity in mice

Data from humans indicate that there is a substantial genetic influence on smoking behavior (Heath, et al., 1995; Kaprio, 2009). A large number of twin studies beginning with those of Fisher (1958) have demonstrated that the heritability of smoking ranges from 0.2 to 0.8 with

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a mean estimate of heritability of 0.53 (Li, 2006). In other words, approximately 50% of the variance of whether an individual is a smoker can be attributed to genetic factors. Genetic factors influence multiple aspects of tobacco use including initiation (Heath and Martin, 1993), persistence (Heath, 1990; Heath and Martin, 1993), number of cigarettes smoked (Carmelli, et al., 1990) and the ability to stop smoking (Carmelli, et al., 1992).

Similarly, the behavioral and physiological effects of nicotine, the major psychoactive component in tobacco, are influenced by genetic factors in mice (for an excellent review see (Marks, 2013)). For example, Marks et al. (1989) demonstrated that there is a 2 to 6-fold difference in ED₅₀ values for a battery of tests for nicotine sensitivity across 19 inbred strains. These tests include nicotine effects on respiration, heart rate, startle response, locomotor activity and body temperature. Miner and Collins (1989) reported similar results for nicotine-induced seizure sensitivity for the same 19 inbred strains. Genetic influences on the development of tolerance to nicotine (Marks, et al., 1986b; Marks, et al., 1991) nicotine oral self-selection (Robinson, et al., 1996; Li, et al., 2007), the effects of nicotine on open field activity (Marks, et al., 1986a) and conditioned place preference (Schechter, et al., 1995; Harenza, et al., 2014) have also been reported. Heritability estimates for some of these behaviors were obtained and found to range from 0.3 for nicotine-induced hypothermia (Marks, et al., 1984) to 0.63 for nicotine-induced seizure sensitivity (Miner, et al., 1984). It does not appear that the heritable differences in response to nicotine in mice are due to variations in nicotine metabolism. For example, Petersen et al. (1984) demonstrated that several mouse strains that vary considerably in their sensitivity to nicotine do not differ in nicotine metabolism. In addition, Miner et al. (Miner, et al., 1984) determined that blood and brain levels of nicotine were not different between the mouse strains C3H/Ibg and DBA/2 following an i.p. injection of nicotine that elicits seizures in C3H/Ibg mice but not in DBA/2 mice.

Although it is clear that natural genetic variation in mice influences sensitivity to nicotine, there has not been an extensive effort to identify the specific genes that contribute to the natural variability in nicotine sensitivity in mice. In this review, those studies that have explored natural genetic variability in mice and its role in nicotine sensitivity will be described. In addition, examples will be provided of how natural genetic variability can confound the results of studies with nicotinic receptor null mutant mice. Such confounds include mice in which the knockout allele has linked genes that have different alleles between the knockout mouse and wild type control and modifier genes that alter the effect of the gene deletion. Being aware of the first confound is essential for interpretation of results from knockout mouse studies while identifying modifier genes may be of value in understanding the neurobiology of nicotine sensitivity.

2.1 Genetic variability in neuronal nicotinic acetylcholine receptor (nAChR) subunit genes

nAChRs are ligand-gated cation channels that are expressed in the brain and periphery (Gahring and Rogers, 2005; Gotti, et al., 2009). The neuronal subfamily consists of the subunits $\alpha 2$ - $\alpha 10$ ($\alpha 8$ exists only in avian species) and $\beta 2$ - $\beta 4$. The nomenclature for the genes that encode the nAChR subunits is *Chrn*xy where *Chrn* stands for cholinergic receptor,

nicotinic, and $\alpha\gamma$ represents the subunit. For example, *Chrna2* is the gene for the $\alpha 2$ subunit. Most effort to identify genetic variants that affect nicotine sensitivity in mice has focused on the nAChR *Chrna7* and *Chrna4* subunit genes. Below is a summary of the findings with these genes as well as other nAChR genes in which natural genetic variability has been associated with sensitivity to nicotine and/or the expression of a given nAChR.

2.2.1 *Chrna7* alleles and $\alpha 7$ expression

The first evidence of genetic variability in mouse nAChR subunit genes was described by Nagavarapu and Boyd (1995). This group identified restriction fragment length polymorphisms (RFLPs) in *Chrna7*, the gene that codes for the $\alpha 7$ nAChR subunit. RFLPs are sequence differences between alleles of a gene that are detected because they either generate or disrupt a recognition sequence for a restriction endonuclease. RFLPs in *Chrna7* subsequently were shown to be linked to individual differences in the level of α -bungarotoxin binding (α BTX), a ligand that specifically labels $\alpha 7$ nAChRs in brain, in various brain regions of an F2 intercross between two mouse strains, C3H/Ibg and DBA/2 (Stitzel, et al., 1996). Interestingly, the linkage of the alleles of *Chrna7* with α BTX levels was found to be brain region specific, with the C3H allele linked to higher levels of α BTX in hippocampus while the DBA/2 allele was linked to higher levels of α BTX expression in striatum. For some brain regions such as cortex, there was no relationship between α BTX levels and *Chrna7* genotype. This brain region-specific effect of *Chrna7* genotype on α BTX levels was confirmed in a later study (Brooks, et al., 2009).

Adams et al. (2001) also examined the role of *Chrna7* genotype on the distribution of α BTX binding in the hippocampus. In this study, Adams et al. utilized a congenic mouse strain in which the DBA/2 allele was introgressed onto a C3H genetic background. Interestingly, results of this study demonstrated that *Chrna7* genotype not only has a quantitative effect on α BTX binding levels but also impacts the neuroanatomical distribution of α BTX-positive hippocampal interneurons. In short, mice that possessed the DBA/2 allele of *Chrna7* exhibited a DBA/2-like distribution of α BTX binding sites irrespective of the genetic background (C3H or DBA/2). Adams further explored the role of *Chrna7* genotype on the developmental profile of α BTX binding during embryonic and perinatal development. In an initial study (Adams, 2003), it was found that C3H and DBA/2 mice possess distinct developmental time courses of $\alpha 7$ expression, with DBA/2 mice showing a later appearance of α BTX binding (embryonic day 16) as compared to C3H mice (embryonic day 13). Using congenic strains in which both alleles of *Chrna7* were exchanged between genetic backgrounds (C3H *Chrna7* allele on a DBA/2 background and DBA/2 allele of *Chrna7* on a C3H genetic background), Adams later reported that the developmental profile of $\alpha 7$ expression was also dependent upon *Chrna7* genotype (Adams, et al., 2006). Mice with the C3H allele of *Chrna7* had an earlier developmental onset of expression irrespective of whether the genetic background was C3H or DBA. Conversely, mice with the DBA/2 allele of *Chrna7* had a delayed onset of $\alpha 7$ expression irrespective of genetic background. The sum of these studies indicates that natural genetic variability in *Chrna7* has a substantial impact on $\alpha 7$ expression in mice at multiple levels, from developmental to anatomical distribution to overall levels. Importantly, these effects appear to be brain region specific.

2.2.2 *Chrna7* alleles and response to nicotine

A few studies have attempted to assess the relationship between natural variability in *Chrna7* in mice and behavioral response to nicotine. In one study, it was reported that the allelic variants of *Chrna7* were linked to individual differences in sensitivity to nicotine-induced convulsions (Stitzel, et al., 1998). These results were consistent with previous work demonstrating that the level of α BTX binding in hippocampus is correlated with nicotine-induced seizure sensitivity (Miner, et al., 1984; Miner, et al., 1985; Miner and Collins, 1989). Nonetheless, further studies are needed to validate the role of *Chrna7* variability in this measure of nicotine sensitivity particularly since deletion of *Chrna7* does not seem to affect seizure sensitivity (Franceschini, et al., 2002) while gain of function increases sensitivity (Broide, et al., 2002).

A recent study by Harenza (Harenza, et al., 2014) utilized recombinant inbred (RI) strains derived from the parent strains C57BL/6 and DBA/2 to assess the relationship between strain differences in α 7 mRNA expression and nicotine place conditioning. This group reported that there was an inverse relationship between α 7 mRNA expression in the striatum and prefrontal cortex of the RI strains and their corresponding place conditioning score. That is, strains that had lower α 7 mRNA expression tended to have greater risk for the rewarding properties of nicotine. Using α 7 knockout mice, it was confirmed that reduced (eliminated) α 7 expression increases nicotine reward as measured by place preference. Also as part of this study, it was reported that there is a provisional cis-expression quantitative trait locus (eQTL) for α 7 expression in the nucleus accumbens. Although the eQTL spans a large region of chromosome 7, it is centered around the *Chrna7* locus suggesting genetic variability in or around *Chrna7* contributes to variability in α 7 expression in the nucleus accumbens. Results also indicated, similar to the results with the previously described C3H and DBA/2 derived mice, that the DBA/2 allele of *Chrna7* is associated with higher levels of α 7 expression in the nucleus accumbens (Brooks, et al., 2009). These findings provide further support for the role of genetic variability in/around *Chrna7* in regulating variability in α 7 expression in mice.

Mouse strain differences in hippocampal α BTX levels also have been implicated in strain variability in auditory gating. Mouse strains with high levels of hippocampal α BTX binding exhibit gating to repeated auditory stimuli while strains with low levels exhibit poor/no gating (Stevens, et al., 1996). Interestingly, the auditory gating deficit in DBA/2 mice, which have low hippocampal α BTX, can be transiently normalized by a single acute injection of nicotine (Stevens and Wear, 1997) or selective α 7 agonists (Stevens, et al., 1998). Because auditory gating correlates with strain differences in hippocampal α BTX levels, it may be linked to strain differences in *Chrna7* genotype. A preliminary analysis using the C3H and DBA/2 congenic strains supports this assumption (Stevens, et al., 2001).

2.2.3 Molecular analysis of *Chrna7* in mice

Mouse *Chrna7* is a relatively large gene at around 135,000 bp and includes 10 exons that code for the canonical α 7 transcript. There are many polymorphisms in *Chrna7* amongst inbred mouse strains. For example, between the mouse strains C3H and DBA/2, there are minimally 493 polymorphisms (data from Mouse Genome Informatics,

www.informatics.jax.org). Between C57BL/6 and DBA/2, there are minimally 232 variations. Determining which of these variants is/are responsible for the relationship between the alleles of *Chrna7* and $\alpha 7$ expression is a daunting task. However, a study by Mexal et al. (2007), did identify two single nucleotide polymorphisms (SNPs) in the promoter region of *Chrna7* between the C3H and DBA/2 alleles of *Chrna7* that affect promoter activity in a cell type specific manner. These two SNPs, in combination, drove higher gene expression in GH4C1 cells if they were of C3H origin, consistent with the relationship between *Chrna7* genotype and $\alpha 7$ expression in the hippocampus (Stitzel, et al., 1996). However, a separate study (Brooks, et al., 2009) reported that *Chrna7* genotype is not linked to $\alpha 7$ RNA levels in adult brain of a C3H by DBA/2 intercross suggesting that the relationship between *Chrna7* genotype and $\alpha 7$ nAChR expression in adult brain is not due to differences in $\alpha 7$ RNA levels, but rather, through a post-transcriptional mechanism. Nonetheless, the in vitro data suggesting a functional role of the *Chrna7* promoter SNPs could be relevant to strain differences in the developmental profile of $\alpha 7$ expression in brain. Further studies are required to address this possibility. In addition, the mechanism responsible for the post-transcriptional effect of *Chrna7* variability of α BTX levels in adult brain should be investigated.

2.3.1 Genetic variability of *Chrna4* in mice

Similar to *Chrna7*, the first evidence of genetic variability in *Chrna4* was provided by the identification of RFLPs between different mouse strains. Utilizing an RI strain panel derived from the mouse lines long sleep (LS) and short sleep (SS), Stitzel et al. (2000) reported that the *Chrna4* RFLP was associated with strain differences in sensitivity to nicotine-induced seizures.

Also using the LS $\times$ SS RI strains, Tritto et al. (2002) found that the *Chrna4* RFLPs were associated with strain differences in the effect of nicotine on locomotor activity, body temperature, respiration rate and startle response. Sequence analysis of cloned cDNAs from the LS and SS mouse lines revealed several SNPs in the coding portion of the $\alpha 4$ subunit (Stitzel, et al., 2001). One of the polymorphisms was a non-synonymous variant that changes a threonine codon to an alanine codon at amino acid position 529 (a T529A variation). Several studies that utilized outbred mice (Tritto, et al., 2001), F2 intercross mice (Li, et al., 2005; Butt, et al., 2005) and inbred strains (Butt, et al., 2005) demonstrated that this T529A variant is linked to differences in free choice oral nicotine intake. Consistent across all studies, mice that possess the T529 allele consumed more nicotine by choice than did mice with the A529 allele.

The effects of the *Chrna4* T529A polymorphism on response to nicotine were further investigated in a transgenic knockin mouse in which the T529A variant was engineered (Wilking, et al., 2010). This mouse allowed the ability to test the specific effect of the T529A polymorphism in the absence of other variants in *Chrna4*. Results from this study confirmed that the T529A variant affects sensitivity to the hypothermic effects of nicotine and free choice nicotine intake. Also reported in this investigation was the observation that the T529A polymorphism significantly impacted conditioned place preference (CPP) for nicotine. At the nicotine dose tested (0.09 mg/kg free base), T529 mice exhibited a

significant place preference while A529 mice displayed no place preference. The sum of these data strongly indicates that the naturally occurring variant in *Chrna4* alters sensitivity to the effects of nicotine in mice. These findings also might indicate why nicotine CPP can be established in C57BL/6 mice, which possess the T529 allele of *Chrna4* while it is difficult, if not impossible, to establish CPP in other strains, such as DBA/2, which possess the A529 allele of *Chrna4* (Grabus, et al., 2006; Harenza, et al., 2014).

It is important to note that mouse strain differences in the neuroanatomical and cell type distribution of the $\alpha 4$ subunit in the hippocampus have been reported (Gahring, et al., 2004). These variations in $\alpha 4$ subunit distribution were found to correlate with strain differences in nicotine induced seizures. However, polymorphisms in *Chrna4* did not account for the altered distribution of $\alpha 4$ subunits across strains. Instead, a locus on chromosome 11 appears to be critical for the differential distribution of hippocampal $\alpha 4$ across mouse strains (Rogers, et al., 2008).

2.3.2 Functional effect of the T529A polymorphism

Both ex vivo studies using mouse brain synaptosomes and in vitro studies using heterologous expression systems have been performed to assess the effect of the *Chrna4* T529 polymorphism on receptor function. $\alpha 4$ almost always pairs with $\beta 2$ and sometimes additional subunits (indicated by *) to form $\alpha 4\beta 2^*$ nAChRs. $\alpha 4\beta 2^*$ nAChRs are known to exist in both high and low sensitivity forms with respect to activation by agonists. These forms of $\alpha 4\beta 2^*$ nAChRs differing in agonist sensitivity are generally believed to be the result of altered receptor stoichiometry (Nelson, et al., 2003; Zhou, et al., 2003). Typically, the high sensitivity form has a stoichiometry of $(\alpha 4)_2(\beta 2)_3$ or $(\alpha 4)_2(\beta 2)_2(\alpha 5)_1$ while the low sensitivity form has a stoichiometry of $(\alpha 4)_3(\beta 2)_2$. Comparison of the function of $\alpha 4\beta 2^*$ nAChRs in synaptosomal preps from mouse strains differing in *Chrna4* T529A genotype indicated that the A529 variant produces greater receptor function per receptor number than the T529 variant at a nicotine concentration that predominantly activates the high sensitivity form of the receptor (Dobelis, et al., 2002). Further, expression of the variant $\alpha 4\beta 2$ nAChRs in a heterologous expression system suggested that the amino acid change in $\alpha 4$ alters the ratio of high to low sensitivity $\alpha 4\beta 2$ nAChRs (Kim, et al., 2003). Wilking et al. (2010) also reported that the T529A variant altered the ratio of high to low sensitivity $\alpha 4\beta 2^*$ nAChRs in synaptosomes prepared from midbrain of the T529A knockin mice.

The finding that the T529A polymorphism alters the ratio of high to low sensitivity $\alpha 4\beta 2$ nAChRs suggests that altering this ratio impacts sensitivity to the effects of nicotine. This observation is consistent with recent data indicating that polymorphisms in human *CHRNA4* that alter the ratio of high to low sensitivity nAChRs are associated with risk for nicotine dependence (McClure-Begley, et al., 2014). Namely, in both humans and mice, polymorphisms that increase the ratio of high to low sensitivity $\alpha 4\beta 2^*$ nAChRs tend to decrease risk for nicotine dependence/nicotine reward.

2.4.1 Genetic variation in *Chrna5* and *Chrna6*

Genetic variants in both *Chrna5* and *Chrna6* also have been reported to be linked to differences in sensitivity to the acute effects of nicotine in mice. A RFLP in *Chrna5*, the

gene that codes for the $\alpha 5$ nAChR subunit, was found to be linked to nicotine-induced seizure sensitivity in the same F2 intercross as described for *Chrna7* (Stitzel, et al., 1998). An RFLP in *Chrna6*, the gene that codes for the $\alpha 6$ nAChR subunit also was linked to nicotine-induced seizure sensitivity in the LSxSS RI lines (Stitzel, et al., 2000). In addition, the *Chrna6* RFLP was associated with strain differences in the effect of nicotine on respiration, acoustic startle, and locomotor activity (Tritto, et al., 2002). The molecular mechanisms through which the variants in *Chrna5* and *Chrna6* might alter nicotine sensitivity remain to be determined. There are no identified non-synonymous mutations in *Chrna5* in mice. However, the inbred strains that were used for the study, C3H and DBA/2 exhibit significant differences in expression of $\alpha 5$ mRNA in the cortex and CA1 region of the hippocampus. These differences are dependent upon *Chrna5* genotype as determined by a congenic strain in which the C3H allele of *Chrna5* was introgressed onto a DBA/2 genetic background (J. Stitzel, unpublished data). Therefore, genetic variants in *Chrna5* that alters $\alpha 5$ expression might be responsible for the linkage between *Chrna5* genotype and nicotine-induced seizures.

With respect to *Chrna6*, there is a non-synonymous mutation that is found in common inbred strains including the LS and SS RI strains. The variant changes a proline codon at position 398 to a serine codon. This variant may be responsible for the association between *Chrna6* genotype and nicotine sensitivity. However, the functional consequence of this polymorphism, if any, is not known.

2.5.1 Genetic variability in other nAChR subunit genes

Table 1 indicates the number of variants identified for the neuronal nAChR subunit genes *Chrna2-Chrna7* and *Chrb2-Chrb4* based on a search of genetic variability for each gene using the Ensembl genome browser (www.ensembl.org). Not surprisingly, polymorphisms exist for every nAChR subunit gene across mouse strains. Most genetic variability is either non-coding (intronic sequence, upstream of the translation initiation codon or downstream of the translation termination codon) or does not alter the amino acid sequence of the encoded protein. However, validated non-synonymous variants have been identified for all but *Chrna3*, *Chrna5* and *Chrna7* (Figure 1). For the non-synonymous SNPs, some have been validated while others have not. Validated non-synonymous SNPs are variants that have been confirmed by a minimum of two separate approaches (e.g., sequencing and SNP-based genotyping) or frequency amongst inbred strains. Some of the non-synonymous variants are found in commonly used inbred mouse strains. These include the non-synonymous variants in *Chrna4* (T529A) and *Chrna6* (P389S) described above which are widely distributed in common mouse strains, as well as S6P and C641Y variants in *Chrna4* that appear to be restricted to Balb/C. There also is a K170R variant in *Chrb3* and a L495P variant in *Chrb4* that are common among standard inbred mouse strains. Because *Chrb3* is physically adjacent to *Chrna6* in the mammalian genome, any association between *Chrna6* genotype and response to nicotine would also implicate genetic variability in *Chrb3*. Therefore, determining whether the non-synonymous *Chrna6* or *Chrb3* variant is functional will be important for understanding the role of these variants in nicotine sensitivity. Similarly, *Chrb4* is part of a gene cluster that includes *Chrna5*. Consequently, it is possible that the linkage observed between *Chrna5* genotype and seizure sensitivity could

be the result of linkage to the *Chrn4* non-synonymous variant because the two strains used to generate the F2 test cross, C3H/Ibg and DBA/2, possess the alternate non-synonymous alleles of *Chrn4*. Again, however, the functional impact of the *Chrn4* L495P variant is not known. Therefore, the relevance of this variant to individual variability in nicotine sensitivity remains to be determined.

All validated non-synonymous variants in *Chrna2*, *Chrna3*, and *Chrn2* are restricted to wild-derived inbred strains. Moreover, a high percentage of the all genetic variation reported in table 1 for the nAChR subunit genes in mice arises from the wild-derived strains. This includes non-coding variants that potentially could affect the spatial, temporal and/or absolute expression of any of the nAChR subunits. Because most current studies on nicotine's effects in mice do not involve wild-derived strains, the genetic variability that is restricted to these strains is of no consequence to the current literature. However, new strains such as those that comprise the collaborative cross inbred strains (Chesler, et al., 2008; Philip, et al., 2011; Chesler, 2014), and the diversity outbred (DO) mice (Svenson, et al., 2012; Chesler, 2014) were developed using a mix of common inbred strains (A/J, C57BL/6J, 129Sv/ImJ, NOD/LtJ, NZO/H1J) and three wild-derived strains (CAST/EiJ, PWK/PhJ, and WSB/EiJ). Because these mouse lines allow for more high resolution genetic mapping and possess significantly more genetic variation among the nAChR subunit genes, it would be of considerable interest to assess the role of nAChR subunit gene variability on nicotine responses in these lines. These lines also are of interest for mapping loci other than those that code for nAChR subunit genes that impact behavioral and physiological responses to nicotine.

3.1.1 Natural genetic variability in nAChR genes and potential interaction with engineered mutations

Extensive work has been done on the effects of nicotine in mice engineered to possess knockout or knockin alleles of most nAChR subunit genes (Picciotto, et al., 2001; Drenan and Lester, 2012; Marks, 2013). Although generally ignored, the natural nAChR variants described in this review potentially could impact the effect of an engineered mutation on nicotine sensitivity. For example, the non-synonymous *Chrna4* T529A variant in mice alters various responses to nicotine including oral nicotine intake and conditioned place preference. Therefore, studies that assess these measures in mice should be cognizant of the *Chrna4* genotype upon which the mutant allele is being assessed.

To better illustrate this point, figure 2 shows data demonstrating that the effect of *Chrna5* deletion on oral nicotine intake is dependent upon *Chrna4* T529A genotype. Oral nicotine intake was measured in F2 intercross mice between C3H/Ibg and C57BL/6 mice possessing the *Chrna5* null mutation. The strains not only differ in the presence/absence of *Chrna5* but also have different alleles of *Chrna4*. C3H mice have the A529 variant of *Chrna4* while C57BL/6 mice carry the T529 allele. Therefore, the two alleles of *Chrna4* segregate independently in the F2 cross as do the normal and null alleles of *Chrna5*. Data from the entire population of F2 mice indicate that *Chrna5* deletion alters nicotine intake, with mice lacking *Chrna5* drinking significantly more nicotine by choice than mice homozygous for the normal allele of *Chrna5*. However, when the F2 population is separated by *Chrna4*

genotype, it is discovered that the effect of *Chrna5* deletion on nicotine intake is dependent upon whether the mice possess the T529 or A529 allele of *Chrna4*. Increased nicotine intake due to *Chrna5* deletion is observed in F2 mice that are homozygous for the T529 allele of *Chrna4*. In contrast, there is no effect of *Chrna5* deletion on nicotine intake in mice homozygous for the A529 allele of *Chrna4*. This observation indicates a genetic interaction between a naturally occurring genetic variation and an engineered mutation. Embracing such interactions and understanding the mechanism responsible for the interaction may be of considerable value in understanding the underlying neurobiology of nicotine sensitivity in mice.

For example, inclusion of an $\alpha 5$ subunit into an $\alpha 4\beta 2^*$ nAChR produces a receptor with high agonist sensitivity (Brown, et al., 2007; Tapia, et al., 2007; Kuryatov, et al., 2008). Likewise, the A529 variant of *Chrna4* leads to an increase in the ratio of high sensitivity to low sensitivity $\alpha 4\beta 2^*$ nAChRs (Wilking, et al., 2010). Because the A529 allele seems to have a similar impact on receptor function as $\alpha 5$, at least in terms of agonist sensitivity, more high sensitivity $\alpha 4\beta 2^*$ receptors may remain in the absence of $\alpha 5$ in an *Chrna4* A529 animal as compared to a T529 animal. If this is the case, then an increase in the ratio of high to low sensitivity $\alpha 4\beta 2^*$ nAChRs, whether it be via insertion of an $\alpha 5$ subunit or an A529 variant of $\alpha 4$, would be protective against nicotine intake and perhaps other drug seeking behaviors. Although this hypothesis is purely speculative, it is testable and also is consistent with a recent publication suggesting that polymorphisms in human *CHRNA4* that increase the ratio of high to low sensitivity nAChRs is protective against nicotine dependence (McClure-Begley, et al., 2014).

3.1.2 Confounding effects of natural genetic variability on the interpretation of results from engineered mutant mice

In addition to directly impacting the results of human-engineered mutations, natural genetic variation in nAChR genes may impact the interpretation of the results obtained from knockout and knockin mice. For example, several studies have reported an effect of *Chrna5* deletion on various measures (Salas, et al., 2003; Kedmi, et al., 2004; Fischer, et al., 2005; Jackson, et al., 2010; Fowler, et al., 2011; Morel, et al., 2014). However, it is important to realize that the *Chrna5* null mutation was made on the *Chrna5* allele of the mouse strain 129SvEv and integrated into the genome of 129SvEv embryonic stem cells (Salas, et al., 2003). Therefore, the null allele of *Chrna5* is flanked by alleles, including those for *Chrna3* and *Chrnb4*, from the 129SvEv genome. Like most engineered mutations, the *Chrna5* null mutation was backcrossed onto a C57BL/6 mouse strain and most studies with the *Chrna5* null mutant mouse have been done on the C57BL/6 background. Relevant to this review, the strains 129SvEv and C57BL/6 possess different alleles for the non-synonymous mutation in *Chrnb4*. Therefore, *Chrna5* null mutant mice on a C57BL/6 background differ not only for the null allele of *Chrna5* but also the non-synonymous allele of *Chrnb4*. Therefore, if the *Chrnb4* non-synonymous polymorphism affects receptor function, the reported effects of *Chrna5* deletion could be due to *Chrna5* deletion, the variant alleles of *Chrnb4*, or potentially both. Some recent studies have confirmed the specific role of *Chrna5* by restoring wild-type behavior via selective re-expression of $\alpha 5$ in the brain of *Chrna5* null mutant mice (Fowler, et al., 2011; Morel, et al., 2014). However, studies in which the

specific role of *Chrna5* has not been validated should be interpreted with some level of caution. It should be noted that the strains used to identify the interaction between the natural *Chrna4*T529A variant and *Chrna5* deletion possess the same allele of *Chrnb4*. Therefore, there is no potential confound relating to *Chrnb4*.

A final example of the potential of natural allelic variation to confound the interpretation of the effects of an engineered mutation arises from a study by Kedmi and Orr-Urtreger (2007). This team reported on the effect of *Chrnb4* gene deletion on the whole mouse brain transcriptome. Like the *Chrna5* null mutation, the *Chrnb4* null mutation was engineered on a 129SvEv background and then crossed into C57BL/6 mice. Results of the study indicated that the two genes that were most down-regulated in the *Chrnb4* null mutant were *Spg21* and *Pts*. It was later revealed that these two genes are near *Chrnb4* on mouse chromosome 9 and 129SvEv and C57BL/6 mice possess different alleles of these genes. Importantly, it was determined that the cause of the reduced expression of these genes in the *Chrnb4* null mutant mouse was most likely the strain-specific alleles of the genes rather than deletion of *Chrnb4*. Without knowledge of the fact that the knockout mouse had different strain alleles flanking *Chrnb4* than the wild type control, it easily could have been concluded that *Spg21* and *Pts* are in some way regulated by *Chrnb4*.

The potential confound of genetic background of the mutant allele on the interpretation of data from mice engineered through homologous recombination, although largely ignored, has been a concern for nearly 20 years (Gerlai, 1996). Moreover, recommendations on how to determine whether an observed effect is due to the manipulated gene or natural variants that flank the mutant allele have been published (Silva, et al., 1997; Gerlai, 2001; Crusio, 2004).

4.1 Relationship between natural genetic variability of the nAChR subunit genes in humans and mice

Humans and mice share orthologs for all nAChR subunit genes. However, no common polymorphisms have been identified between the orthologous nAChR subunit genes in humans and mice. Despite this fact, the genetic variants that have been studied in mice have provided useful information with respect to interpreting human genetic data. As previously described in this review, several rare mutations in human *CHRNA4* (symbols for human genes are all capital letters) have been associated with reduced risk for nicotine dependence. Based on in vitro studies with the various rare mutations in human *CHRNA4*, it was postulated that polymorphisms that increase the ratio of high to low sensitivity $\alpha 4\beta 2$ nAChRs decrease risk for nicotine dependence (McClure-Begley, et al., 2014). Results from the mouse *Chrna4* T529A polymorphism strongly support this possibility. This polymorphism, which increases the ratio of high to low sensitivity $\alpha 4\beta 2^*$ nAChRs in the midbrain, decreases nicotine place preference and reduces nicotine intake in mice (Wilking, et al., 2010). Thus, despite the fact that the T529A variant is unique to mice, it provides direct evidence for a relationship between a polymorphism that alters the ratio of high to low sensitivity $\alpha 4\beta 2^*$ nAChRs and nicotine reward and intake.

In the case of human *CHRNA7*, genetic variability in *CHRNA7* as well as individual differences in the expression of hippocampal $\alpha 7$ nAChRs have been associated with auditory gating deficits and/or schizophrenia (Leonard and Freedman, 2006). In addition, polymorphisms in human *CHRNA7* that lead to decreased promoter activity are associated with increased risk for schizophrenia (Leonard, et al., 2002). As described above, studies with mice also have shown that genetic variability in *Chrna7* is linked with reduced expression of $\alpha 7$ in the hippocampus (Stitzel, et al., 1996;Stitzel, et al., 1998;Brooks, et al., 2009) and deficits in auditory gating (Stevens, et al., 2001). Moreover, polymorphisms in mouse *Chrna7* that decrease promoter activity (Mexal, et al., 2007) are associated with reduced $\alpha 7$ expression and deficits in auditory gating. Again, despite the fact that the polymorphisms are unique between human and mouse *CHRNA7/Chrna7* genes, the consistency of the impact of the variants on receptor expression and behavior between humans and mice strengthens the findings from both species.

5.1 Other natural genetic variability and nicotine responses in mice

Only a few studies that have attempted to identify chromosomal loci other than nAChR subunit genes that are linked to nAChR expression and/or response to nicotine. As previously mentioned, Rogers et al. (2008) reported that a locus on chromosome 11 is critical for mouse strain differences in the spatial and cell-type specific expression of the $\alpha 4$ subunit in the hippocampus, a measure that correlates with sensitivity to nicotine induced seizures. Gill and Boyle (Gill and Boyle, 2005;Boyle and Gill, 2009) also reported loci on chromosomes 2, 11, 14, 16 and 17 that influence the effects of nicotine on locomotor activity. Finally, Li et al., (2007) described loci on chromosomes 1, 4, 7 and 15 that account for approximately 60% of the phenotypic variability in oral nicotine intake. Although no specific genetic variants have been identified that are responsible for the association of these chromosomal regions and measures on nicotine sensitivity/nAChR expression, none of the identified loci for any of the described studies map to locations of nAChR subunit genes. Clearly, genes other than those that code for nAChR subunit genes also contribute to mouse strain differences in response to nicotine and/or nAChR expression. Unfortunately, standard genetic mapping approaches have limited ability to identify the specific genetic variants that contribute to complex phenotypes such as response to nicotine. However, utilizing animals from the collaborative cross, the diversity outbred cross, or other mouse populations such as HS/Npt mice (Demarest, et al., 2001) may allow for higher resolution mapping of genes that influence sensitivity to those effects of nicotine that are testable in mice. Such a higher resolution mapping could lead to the identification of novel genes that contribute to individual variability in response to nicotine. Moreover, crossing a nAChR null mutant to one of these high resolution mapping lines could lead to the identification of novel modifier genes that would enhance our understanding of the mechanism through which null mutation alters nicotine sensitivity.

6.1 Summary

Naturally occurring genetic variability in nAChR subunit genes as well as polymorphisms in other less well defined loci clearly contribute to variability in nicotine sensitivity and nAChR expression in mice. Studies on natural variants in *Chrna4* and *Chrna7* have

provided unique insights into the impact of genetic variability on nicotinic receptor expression, function and sensitivity to nicotine. Identifying and characterizing the functional variants of other nAChR subunit genes and non-nAChR genes associated with the effects of nicotine in mice will improve our understanding of the neurobiology of nicotine sensitivity. Importantly, studies that utilize mice with engineered mutations should not ignore the impact of natural genetic variability on nicotine sensitivity and/or nAChR expression. This is especially the case when the engineered mutation was made on a different genetic background than the strain in which it is being tested. Finally, embracing natural genetic variability and its potential to alter the effect of the mutation on nicotine sensitivity may provide novel opportunities to identify modifier genes that will enhance our understanding of the mechanism through which the engineered mutation alters nicotine sensitivity.

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Polymorphisms exist for all nicotinic receptor subunit genes in mice

Chrna4, Chrna5, Chrna6 and Chrna7 variants are linked to nicotine sensitivity in mice
Functional effects of polymorphisms in Chrna4 and Chrna7 have been identified
Genetic variability in other unidentified genes alters nicotine sensitivity in mice
Natural genetic variability can confound or modify the effect of engineered mutations

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Figure 1.

Schematic representation of the location of all validated non-synonymous polymorphisms identified in the neuronal nAChR genes *Chrna2-Chrna7* and *Chrb2-Chrb4*. Variants found in at least one common inbred strain is indicated by ▽. Polymorphisms restricted to wild-derived strains are indicated by ▼. *C641Y variant in *Chrna4* is located in only one of the two known full-length splice variants of *Chrna4*. Data were extracted from either the Mouse Genomes Project or the Perlegen MM Panel2 genotyping sample as available on the Ensembl genome browser (<http://www.ensembl.org>). Common inbred strains screened for polymorphisms include 129P2/OlaHsd, 129S1/SvImJ, 129S5SvEvBrd, A/J, AKR/J, BALB/cJ, BALB/CByJ, BTBR T+ TF/J, C3H/HeJ, C57BL/6NJ, CBA/J, DBA/2J, FVB/NJ, KK/HlJ, LP/J, NOD/LtJ, NOD/ShiLtJ, NZO/HlLtJ, and NZW/LACJ. Wild-derived strains screened for polymorphisms include CAST/EiJ, MOLF/EiJ, PWK/PhJ, PWD/PhJ, SPRET/EiJ, and WSB/EiJ. In some cases, not every strain listed was screened for a given variant. S = signal peptide, TM1-4 represent the approximate location of the four transmembrane domains present in nAChR subunit proteins. □ = cysteine loop present in all nAChR subunits. ▢ = vicinal cysteine residues of α subunits.

Figure 2.

The effect of *Chrna5* deletion on oral nicotine intake is dependent upon natural variant alleles in *Chrna4*. *Chrna5* deletion increases oral nicotine intake in an F2 intercross between C3H/1bg mice and C57BL/6 mice harboring the *Chrna5* null mutation ($F(1,161) = 22.95$, $p < 5 \times 10^{-6}$) (A). When the analysis is restricted to F2 mice possessing the T529 allele of *Chrna4*, *Chrna5* deletion also leads to increased nicotine intake ($F(1, 27) = 11.6$, $p < 5 \times 10^{-3}$). However, the effect of *Chrna5* deletion on nicotine intake is abolished in F2 mice homozygous for the A529 allele of *Chrna4* ($F(1,22) = 0.469$, $p > 0.5$). All data were analyzed via repeated measures ANOVA. Both female and male mice were used. However, no main effect of sex or interaction between sex and genotype were observed. Therefore, data are collapsed on sex.

Table 1

Genetic variation in the nAChR subunit genes in inbred mouse strains

Gene	Number of identified variants	Non-synonymous variants (Total/Validated)	Non-synonymous variants found in at least one common inbred strain	Non-synonymous variants restricted to wild-derived inbred strains
<i>Chrna2</i>	636	7/3	0	7/3
<i>Chrna3</i>	716	1/0	0	1/0
<i>Chrna4</i>	2374	8/5	3/3	5/2
<i>Chrna5</i>	1020	7/0	0	7/0
<i>Chrna6</i>	743	8/3	1/1	7/2
<i>Chrna7</i>	3997	0	0	0
<i>Chrn2</i>	418	3/2	0	3/2
<i>Chrn3</i>	3092	6/3	1/1	5/2
<i>Chrn4</i>	927	5/1	3/1	2/0