



Translational Research in Nicotine Dependence

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Nicotine addiction accounts for 4.9 million deaths each year. Furthermore, although smoking represents a significant health burden in the United States, at present there are only three FDA-approved pharmacotherapies currently on the market: (1) nicotine replacement therapy, (2) bupropion, and (3) varenicline. Despite this obvious gap in the market, the complexity of nicotine addiction in addition to the increasing cost of drug development makes targeted drug development prohibitive. Furthermore, using combinations of mouse and human studies, additional treatments could be developed from off-the-shelf, currently approved medication lists. This article reviews translational studies targeting manipulations of the cholinergic system as a viable therapeutic target for nicotine addiction.

Smoking is the cause of 4.9 million deaths each year (Ebbert et al. 2010). Although the detrimental effects of smoking are commonly understood and 70% of smokers indicate that they want to quit, successful attempts among smokers remain below 5% (Nides 2008). Many factors contribute to the development and maintenance of nicotine addiction, and these processes have been investigated in both human populations and animal models to better understand underlying mechanisms. Cessation of chronic nicotine produces withdrawal symptoms in both animals (Grabus et al. 2005; Malin et al. 1992) and humans (Hughes 2007; Hendricks et al. 2006), and avoidance of withdrawal symptoms is one factor that contributes to the maintenance of smoking and relapse during quit attempts. Conversely, studies have also shown that the severity and duration of nicotine withdrawal symptoms strongly predict relapse (Piasecki

et al. 1998, 2000). Therefore, recent endeavors in medication development are targeted at attenuation of nicotine withdrawal symptoms.

Currently, there are three “first-line” pharmacotherapies for nicotine addiction: nicotine replacement therapy, bupropion (a mixed norepinephrine/dopamine reuptake inhibitor), and varenicline (a nicotinic acetylcholine receptor [nAChR] partial agonist). The most promising of the current pharmacotherapies is varenicline (Chantix), which results in maintenance of smoking cessation in nearly 80% of treatment-seeking participants (Cahill et al. 2011). The success of varenicline, which targets $\alpha 4\beta 2$ nAChRs, suggests that compounds modulating the cholinergic system and/or nicotinic receptors would serve as promising drugs for utilization as a smoking cessation therapy. Therefore, the central theme of this translational article focuses on modulation of the cholinergic system

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as a viable candidate for smoking cessation treatment, whether this be through modulation of the endogenous system via synthesis and release or through the direct activation of nAChRs.

NEUROBIOLOGY UNDERLYING THE ADDICTIVE NATURE OF NICOTINE

Neurochemistry of Nicotine

Nicotine, the major psychoactive component in tobacco smoke, is thought to mediate both tobacco reinforcement and dependence (Le Foll and Goldberg 2006). Once in the bloodstream, nicotine, a highly lipophilic compound, rapidly crosses the blood–brain barrier (Gahring and Rogers 2005) and can be sequestered in lipid-rich, slightly basic reservoirs, such as glia (Crooks 1999). This compartmentalization of nicotine can lead to its accumulation in the brain during chronic administration (Ghosheh et al. 2001), potentially producing continued effects following termination of nicotine exposure. Once in the brain, nicotine binds to nAChRs. These receptors are pentameric ion channels, which pass Na^+ , K^+ , and Ca^{2+} ions and thus have the ability to alter cellular activity. Entry of these ions can either directly impact cell excitability or trigger calcium-sensitive molecules, such as protein kinase C (PKC) (Soliakov and Wonnacott 2001), protein kinase A (PKA) (Dajas-Bailador et al. 2002), calmodulin-dependent protein kinase II (CAMKII) (Steiner et al. 2007), and extracellular signal-regulated kinases (ERKs) (Dajas-Bailador et al. 2002; Steiner et al. 2007). These calcium-sensitive kinases then have myriad downstream effects, including activation of transcription factors such as CREB (Chang and Berg 2001; Pandey et al. 2001; Hu et al. 2002; Brunzell et al. 2003; Walters et al. 2005) (for a review of signaling effects of nicotine, see Shen and Yakel 2009).

Nicotinic receptors come in two moieties, homomeric receptors (e.g., $\alpha 7$) or heteromeric receptors (e.g., $\alpha 4\beta 2$) (for more information on specific subtypes, see Fowler et al. 2008). However, in this work, we will be focusing on the $\alpha 4\beta 2$ heteromeric receptor in terms of its translational importance. In contrast to other

subtypes, the $\alpha 4\beta 2$ nAChR subtype has been shown to readily up-regulate following chronic exposure to nicotine, as evidenced by findings in cell culture (Xiao and Kellar 2004), rodents (Schwartz and Kellar 1983; Marks et al. 1985), monkeys (Picciotto et al. 2008), and humans (Mukhin et al. 2008). The up-regulation of receptors in the face of increased agonist is a characteristic unique to nicotine, and its full importance is not understood. However, PET imaging in human smokers following different periods of abstinence suggest that this up-regulation may directly contribute to smoking relapse. Cosgrove and colleagues showed that $\beta 2$ -containing nAChRs remain significantly up-regulated after 1 mo of abstinence and their density was positively correlated to craving (Cosgrove et al. 2009). Although correlational, this study suggests that the phenomenon of receptor up-regulation could directly contribute to failed smoking cessation.

Cholinergic System: Synthesis, Release, and Activation of nAChRs

Nicotinic receptors are endogenously activated through the binding of acetylcholine (ACh) (Fig. 1). The rate-limiting step in the synthesis of acetylcholine (ACh) is the availability of choline in the cell (Jope 1979). Therefore, cholinergic cells possess a high-affinity choline transporter (HA-TS) on their terminals, in addition to the low-affinity choline transporter found on all neuronal cell types (Simon and Kuhar 1975; Okuda et al. 2000). ACh is actively synthesized via choline acetyltransferase (ChAT), which is located in the presynaptic nerve terminals near synaptic vesicles (Jope 1979). Because this enzyme is exclusive to the synthesis of ACh, it is a reliable marker of cholinergic neurons (Eckstein and Sofroniew 1983). Cholinergic neurons can be subdivided into two major pools: (1) projection neurons arising from cholinergic nuclei and (2) subpopulations of cholinergic interneurons (Hoover et al. 1978). The major cholinergic projection neurons in the brain arise from two main sets of nuclei: the basal forebrain cholinergic complex, which includes the nucleus basalis, diagonal band of Broca, and

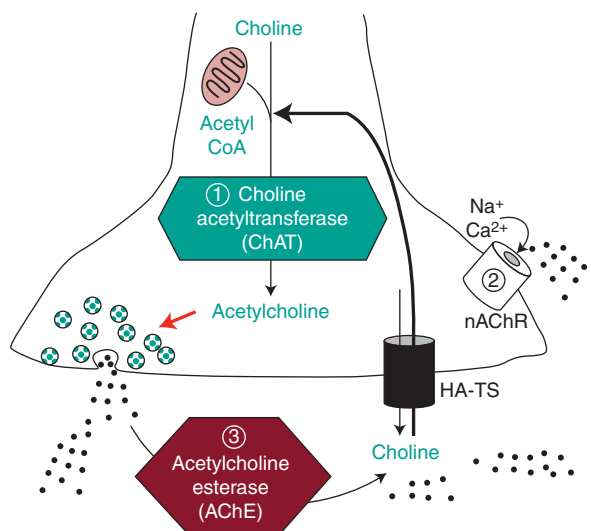


Figure 1. Diagram of endogenous cholinergic transmission in the brain. Choline acetyltransferase (ChAT, 1) enzymatically produces acetylcholine from the precursors choline and acetyl CoA. Following synthesis, acetylcholine is packaged in presynaptic vesicles and released, where it can act on nicotinic acetylcholine receptors (nAChR, 2). Termination of acetylcholine activity is accomplished via enzymatic breakdown by acetylcholinesterase (AChE, 3), yielding choline and acetate. Choline is then actively transported back into the cell by the high-affinity choline transporter (HA-TS) and resynthesized into acetylcholine. Viable smoking cessation drug targets in the cholinergic system include synthesis (1), receptor activation (2), and degradation (3).

medial septal nuclei and the pontomesencephalotegmental cholinergic complex, which includes the pedunculopontine nucleus and dorsolateral tegmental nuclei (Hoover et al. 1978; Eckenstein and Sofroniew 1983). Projections arising from these cholinergic nuclei innervate diverse brain areas, and thus impinge on numerous neural networks controlling such states as cognition, reward, and affect (Johnston et al. 1979; Muir et al. 1993). The second major pool of acetylcholine in the central nervous system (CNS) arises from cholinergic interneurons (Hoover et al. 1978). These GABAergic cells, which have been most thoroughly investigated in the cerebellum (Kalnichenko and Okhotin 2005), hippocampus (Lawrence 2008; Drever et al. 2011), and striatum (Oldenburg and Ding 2011), corelease ACh during high frequency stimulation, leading to strong modulation of local circuitry within the specific brain regions (Witten et al. 2010). Following release of ACh from either cholinergic projection neurons or cholinergic interneurons, ACh is inactivated through rapid degradation in the synapse by the enzyme acetylcholinesterase

(AChE), rather than reuptake as is the case with many neurotransmitters (Zimmerman and Soreq 2006).

Although cholinergic projection neurons and interneurons are widely distributed throughout the CNS, nAChRs are predominantly found on presynaptic terminals. Thus, the main effect of nicotine is to modulate the release of numerous other neurotransmitters, including serotonin, norepinephrine, dopamine, acetylcholine, glutamate, and GABA (for a review, see Wonnacott et al. 2006). This presynaptic modulatory role of nicotinic receptors in the CNS may in fact underlie many of the problems in developing a successful smoking cessation aid. For example, in order for a pharmacotherapy to be successful, it needs to address not only central nicotinic signaling, but also nicotine's potential effects on diverse other neurotransmitter systems. Further complicating this issue is that knowledge of how the cholinergic system is altered in a smoker remains largely unknown. Therefore, examination of human and animal studies investigating alterations to or polymorphisms

within the cholinergic system may lead to more effective novel therapeutics for nicotine dependence.

TRANSLATIONAL STUDIES TARGETING MANIPULATIONS OF THE CHOLINERGIC SYSTEM

Acetylcholine (ACh) Synthesis Pathway—Rate-Limiting Steps and Genetic Influences

ChAT Studies in Smokers

Genetic studies in smokers have found significant association of single-nucleotide polymorphisms (SNPs) in the ChAT gene with smoking cessation phenotypes. An initial pharmacogenetic study found that a single ChAT SNP (rs1917810) was associated with higher abstinence rates in smokers with the minor allele (Heitjan et al. 2008). Follow-up studies in both treatment-seeking smokers (Ray et al. 2010) as well as nontreatment seekers (Wei et al. 2010) also showed significant association of ChAT SNPs or haplotypes with multiple measures of smoking and nicotine dependence. In the treatment seekers, a systems-based genetic association analysis was performed assessing smoking relapse following 8 wk of transdermal nicotine therapy. This approach highlighted a cluster of ChAT SNPs that were significantly associated with relapse (Ray et al. 2010). A replication and extension of this finding in non-treatment seekers examined SNPs in the ChAT gene for association with a number of nicotine dependence parameters, including smoking quantity and heaviness of smoking. This study, which used a haplotype-based association analysis, identified several ChAT haplotypes that were significantly associated with all parameters of nicotine dependence (Wei et al. 2010). In total, these human studies support additional investigation of ChAT manipulations for potential smoking cessation therapeutics.

ChAT Studies in Rodents

Because of the link between human SNPs in the ChAT gene and smoking behavior, it could be hypothesized that endogenous cholinergic tone

afforded by ChAT activity may influence nicotine withdrawal behaviors. Indeed, animal studies probing the mechanistic effects of chronic nicotine during adolescence or adulthood have shown long-term alterations of ChAT activity (Abreu-Villaca et al. 2003; Ribeiro-Carvalho et al. 2009). Specifically, chronic exposure to nicotine during adolescence was shown to increase ChAT activity in adulthood in the cortex and hippocampus of male rodents (Abreu-Villaca et al. 2003; Ribeiro-Carvalho et al. 2009). This effect appears to be sex specific, because chronic nicotine treatment in adolescent female rodents results in a decreased ChAT activity profile during adulthood when compared to untreated female littermates (Ribeiro-Carvalho et al. 2008). However, it remains unknown whether these alterations are driven hormonally or through inherent sex differences in the cholinergic system.

In addition to adolescent studies, it has also been shown that chronic nicotine treatment during adulthood impacts ChAT activity as well. Adult exposure in male rodents resulted in decreased ChAT activity in the midbrain (Ciani et al. 2005), striatum, and cortex (Slotkin et al. 2008) following chronic nicotine treatment. However, following 72 h of nicotine withdrawal, ChAT activity was elevated in males, but not females (Slotkin et al. 2008), suggesting a homeostatic response in males but not females to the nicotine withdrawal state. This finding, in addition to the sex-specific effects observed following adolescent exposure (Ribeiro-Carvalho et al. 2009), may contribute to the differential nicotine reward and withdrawal profiles reported in women (for a review, see Perkins et al. 1999). The combined observations of rodent ChAT studies and human ChAT SNP studies suggest that modulation of endogenous ACh may be a viable candidate for future smoking cessation medication development.

Potential Therapeutics

Increased ACh Synthesis via Dietary Supplementation. Long-lasting alterations in the cholinergic system may indicate a need for increased modulation of the endogenous cholinergic

system in treatment of nicotine dependence and withdrawal. Manipulations of acetylcholine synthesis via dietary choline supplementation have been shown to increase nAChR-binding sites in the hippocampus, frontal cortex, and striatum to levels similar to that seen following chronic nicotine administration (Coutcher et al. 1992). Furthermore, dietary supplementation with choline or other nutrients, like acetyl-L-carnitine and huperzine A, which have also been shown to increase acetylcholine levels in the brain, have been shown to rescue cognitive impairments in a number of animal models (De Bruin et al. 2003; Teather and Wurtman 2005, 2006; Wang et al. 2007; Holguin et al. 2008a,b). Although a clinical report in healthy young subjects did not report any significant effects on cognitive performance following dietary choline supplementation (Deuster et al. 2002), nutrient supplementation targeting increased synthesis of acetylcholine in persons with traumatic brain injury reported significant improvements in general cognitive functioning (Amen et al. 2011). These data suggest the benefit of increased ACh is evident only under conditions in which deficits are present. A clinical hallmark of nicotine withdrawal is cognitive impairment; therefore, these animal and human studies suggest that modulation of central acetylcholine levels via dietary supplementation may be a valid adjunctive treatment during smoking cessation.

Increased ACh Levels via Acetylcholinesterase Inhibitors. Because the actions of ACh in the synapse are terminated by degradation of the neurotransmitter by the enzyme acetylcholinesterase (AChE), an alternative approach to increase cholinergic tone in the synapse is through administration of AChE inhibitors. Inhibition of AChE is the primary mode of action of rivastigmine, donepezil, and galantamine, all of which are commonly prescribed medications for cognitive impairment and first-line medications for Alzheimer's disease (Mehta et al. 2012). In nicotine-dependent animals, studies utilizing these drugs during withdrawal show that increasing endogenous ACh tone via inhibition of AChE can rescue some aspects of cognitive withdrawal deficits (Wilkinson and Gould

2011). Furthermore, although galantamine has not been successful at reducing smoking behavior in schizophrenic patients (Kelly et al. 2008), treatment with galantamine in alcohol-dependent smokers had promising results (Diehl et al. 2006).

nAChR Signaling—Polymorphisms in Subunits and Subsequent Downstream Effects

nAChR Polymorphism Studies in Smokers

Polymorphic alterations in the subunits comprising nAChRs can also impact smoking behaviors. Whether using a candidate gene approach or a genome-wide association studies (GWAS) approach, a number of studies have implicated SNPs occurring in nicotinic subunit genes in the etiology of smoking. The most widely evaluated example of this is the CHRNA5-CHRNA3-CHRNA4 gene cluster, which has been examined for associations with nicotine dependence phenotypes, withdrawal symptoms, and smoking cessation. A number of recent GWAS and pathway-based studies have identified SNPs in this gene cluster associated with heaviness of smoking and/or nicotine dependence (Berrettini et al. 2008; Bierut et al. 2007; Caporaso et al. 2009; Saccone et al. 2007; Thorgeirsson and Stefansson 2008; Thorgeirsson et al. 2010). Other aspects of nicotine dependence, such as nicotine tolerance, craving, withdrawal severity, and inability to stop smoking, have also been associated with CHRNA5-CHRNA3-CHRNA4 SNPs (Baker et al. 2009). However, although this highly reproducible finding implicates this gene cluster in various aspects of nicotine dependence, whether it predicts cessation rates is unclear. For example, in both retrospective and prospective studies no evidence was found for associations of SNPs in the CHRNA5-CHRNA3-CHRNA4 gene cluster with smoking cessation (Conti et al. 2008; Breitling et al. 2009; Ray et al. 2010). Yet, two SNPs in the CHRNA5 and CHRNA4 genes were identified as predictors of abstinence at 52-wk followup (Sarginson et al. 2011), and in pharmacogenetic studies, SNPs in this gene cluster appear to predict

success rates with either nicotine or varenicline treatment (Munafò et al. 2011; King et al. 2012).

Other nicotinic subunits have also been implicated in nicotine dependence. For example, SNPs in *CHRNA6* and *CHRNA3*, the genes encoding the $\alpha6$ and $\beta3$ subunits, have been associated with nicotine dependence, subjective response to nicotine, and self-reported number of unsuccessful quit attempts (Bierut et al. 2007; Greenbaum et al. 2006; Saccone et al. 2007; Zeiger et al. 2008; Hoft et al. 2009). Additional studies have implicated the gene encoding for the $\alpha4$ subunit, *CHRNA4*, in various smoking phenotypes (Breitling et al. 2009; Wessel et al. 2010; Han et al. 2011; Xie et al. 2011). Furthermore, polymorphisms in the *CHRNA4* gene have been significantly associated with abstinence rates while on nicotine replacement therapy (Hutchison et al. 2007) or varenicline (King et al. 2012), suggesting a potential role for *CHRNA4* as a target for therapeutic intervention.

nAChR Polymorphism Studies in Rodents

Studies from nicotinic receptor knockout mice have helped to elucidate the relative contributions of specific subunits to discrete behaviors pertinent to nicotine dependence and withdrawal (for a review, see Fowler et al. 2008). For example, $\beta2$ knockout mice do not show nicotine self-administration (Picciotto et al. 1998), nor do they show many of the classical behavioral responses to nicotine (Shoib et al. 2002; Besson et al. 2006; Walters et al. 2006). However, the dramatic alterations inherent to complete loss of a nAChR subunit are not likely to reflect the general smoking population. Therefore, in terms of translational relevance, studies of rodent polymorphisms in nAChR subunits may be more representative of the repercussions of nAChR SNPs in the clinical population.

As an example of this, there is a missense polymorphism in the mouse *Chrna4*, which results in altered receptor function, including regulation of receptor stoichiometry (Dobelis et al. 2002; Kim et al. 2003). Studies using multiple mouse strains with differing genotypes at this polymorphism show that this SNP influences

both nicotine preference and consumption in mice (Butt et al. 2005). To confirm that this SNP is sufficient for the behavioral alterations, studies using a knockin mouse with this same polymorphism showed that the threonine to alanine substitution results in reduced nicotine consumption, reduced nicotine reward, as well as altered $\alpha4\beta2^*$ receptor function (Wilkings et al. 2010). Although the equivalent of this SNP has not been identified in humans, these studies highlight the importance of examining natural occurring variation, in which a single-nucleotide alteration results in significant behavioral effects, in lieu of knockout mice or overexpression systems that may not be representative of human genetic variation. Furthermore, given the altered receptor function or altered stoichiometry arising from these murine SNPs, a similar situation may underlie the $\alpha4$ SNPs and their associated phenotypes in human studies. However, further studies are necessary to directly evaluate this possibility.

To date, there are no mouse models that recapitulate variants of the human SNPs for the *CHRNA5-CHRNA3-CHRNA4* gene cluster, or for the *CHRNA6*, *CHRNA4*, and *CHRNA3* receptors. To approach this knockin mice could be generated that specifically harbor the human SNPs, or the mouse equivalent of these SNPs. The generation of such mice would allow for the examination of molecular mechanisms that mediate the associated behavioral consequences and thus contribute to a better understanding of their impact in nicotine dependence.

Potential Therapeutics

Coordinated Pharmacogenetic Cessation Treatment. Pharmacogenetic approaches to smoking cessation attempt to individualize treatment through the tailoring of a person's therapy with their genetic information. Currently, the most successful pharmacotherapy for smoking cessation is varenicline (Chantix), an $\alpha4\beta2$ nicotinic partial agonist. However, its efficacy rates appear to be variable in the general population. This variability could in fact be owing to differential effects in persons with polymorphisms in the genes encoding for

varenicline's target nicotinic receptor subunits, $\alpha 4$ and $\beta 2$. In fact, a recent clinical trial investigating the pharmacogenetics of smoking cessation aids showed that continuous abstinence rates while on varenicline treatment is highly associated with SNPs in these subunits (King et al. 2012). Although perhaps economically unfeasible at present, this avenue of treatment may be more realistic in the future, with increased cost efficiency of individualized genotyping owing to higher throughput and improved methodology in genetic sequencing technology (Wu and Fuhlbrigge 2008; Olgati et al. 2012). Furthermore, pharmacogenetic information is currently included with over 60 FDA-approved drugs (Frueh et al. 2008), indicating a progressive shift in the applicability of this approach. Therefore, further translational investigations highlighting gene by drug interactions may prove highly beneficial in the future.

CONCLUDING REMARKS

The cost of designed drug discovery is \$1.8 billion dollars per drug, with a multitude of failed drugs along the way (Paul et al. 2010). Thus, investing in new approaches to bridge animal and human studies to evaluate novel therapeutics could significantly improve success rates. Furthermore, using combinations of mouse and human studies, additional treatments could be developed from off-the-shelf, currently approved medication lists. For example, bupropion is an antidepressant originally developed for the treatment of mood disorders. Numerous studies have shown a high degree of comorbidity between mood disorders and nicotine dependence, which supported bupropion's utility as a smoking cessation aid (Totterdell 2006). This logical extension proved rewarding, as it is currently the second most efficacious smoking cessation aid on the market after varenicline. There is a similar case for therapeutics used in other disease states. For example, nicotine is protective against Alzheimer's disease and other forms of dementia (for a review, see Shimohama and Kihara 2001). However, the novel application of cognitive impairment therapeutics for the treatment of nicotine dependence is

gaining traction with increased animal and human studies evaluating cognitive enhancing drugs for smoking cessation. Perhaps future studies examining the underlying benefits of or vulnerabilities to nicotine within other comorbid populations will prove valuable in developing new smoking cessation therapies. However, this type of coordinated understanding will only be attainable through increased translational studies and communication between the animal and human side of nicotine dependence research.

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