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# From Smoking to Lung Cancer: The CHRNA5/A3/B4 Connection

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## Abstract

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that modulate key physiological processes ranging from neurotransmission to cancer signaling. These receptors are activated by the neurotransmitter, acetylcholine, and the tobacco alkaloid, nicotine. Recently, the gene cluster encoding the α3, α5 and β4 nAChR subunits received heightened interest after a succession of linkage analyses and association studies identified multiple single nucleotide polymorphisms (SNPs) in these genes that are associated with an increased risk for nicotine dependence and lung cancer. It is not clear whether the risk for lung cancer is direct or an effect of nicotine dependence, as evidence for both scenarios exist. Here, we summarize the body of work implicating nAChRs in the pathogenesis of lung cancer, with special focus on the clustered nAChR subunits and their emerging role in this disease state.

### Keywords

Nicotine Addiction; Lung Cancer; Nicotinic Acetylcholine Receptors; CHRNA5/A3/B4 gene cluster

## Introduction

Tobacco use is the leading cause of preventable mortality around the world, resulting in more than 5 million deaths per year (WHO 2009). Approximately 600,000 of these deaths are due to second-hand smoke, with one third of the adult population exposed to second-hand smoke globally. In the United States, overall tobacco use has been declining but approximately 46 million adults still smoked in 2008 (CDC 2009). If current trends persist, tobacco may kill a billion people by the end of this century.

The list of diseases caused by tobacco use is expanding, according to a recent Surgeon General's report on the health effects of smoking (HHS 2004). A causal relationship was reported between active smoking and cardiovascular diseases, respiratory diseases, reproductive disorders, and several types of cancers including cancers of the lung, bladder, cervix, esophagus, kidney, larynx, mouth, pancreas, stomach as well as leukemia.

Cigarette smoke contains 4000 chemicals, 250 of which are known to be harmful, and at least 50 of which are carcinogens (Shields 2002). The most potent of these carcinogens are polycyclic aromatic hydrocarbons and nicotine metabolites such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN). These nitrosamines form DNA adducts that cause mutations leading to cancer (Hecht and

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Hoffmann 1988). In the following sections, we review evidence accumulated through the years (see Timeline) showing that nicotine, itself, promotes lung cancer through its interaction with nAChRs.

## Nicotinic acetylcholine receptors

nAChRs are a heterogeneous family of ligand-gated cation channels activated by the endogenous neurotransmitter acetylcholine (ACh) and exogenous chemicals such as nicotine and its metabolites. nAChRs were the first receptors to be characterized at the biochemical, biophysical, molecular, and pharmacological levels and have served as prototypes for all other ligand-gated ion channels including those activated by 5-hydroxytryptamine (5-HT3), γ-aminobutyric acid ($GABA_A$ and GABAC), and glycine (Le Novere and Changeux 1995, Taly et al 2009). Ligand binding induces a conformational change causing the channel to open thereby allowing the flow of $Na^+$, $K^+$, and $Ca^{2+}$ ions down their electrochemical gradients. The propensity of nAChRs to flux intracellular calcium levels is important in the activation of downstream signaling cascades (Fucile 2004).

nAChRs can be classified into two main categories: muscle or neuronal receptors. Muscle nAChRs are expressed primarily in skeletal neuromuscular junctions and are composed of the α1, β1, δ, and ε or γ subunits (McGehee and Role 1995). In contrast, neuronal nAChRs were originally cloned from neuronal-like cell lines and brain cDNA libraries, hence their name, and are expressed throughout the nervous system where they increase neuronal excitability and facilitate synaptic transmission (Albuquerque et al 2009, Dani and Bertrand 2007, McGehee and Role 1995). Twelve neuronal nAChR subunits have been identified, namely α2-α10 and β2-β4 (Boyd 1997, Gotti et al 2006, Patrick et al 1993). Expression of these subunits has also been observed in many other cell types including endothelial cells, gastrointestinal tissue, glia, immune cells, keratinocytes, and lung tissue (Arredondo et al 2001, Battaglioli et al 1998, Gahring et al 2004, Gahring and Rogers 2006, Kawashima and Fujii 2003, Macklin et al 1998, Maus et al 1998, Nguyen et al 2000, Spindel 2003, Wang et al 2001, Wessler and Kirkpatrick 2008).

nAChRs are integral membrane proteins composed of five subunits symmetrically arranged around a central pore (Figure 1A) (Corringer et al 2000). Each nAChR subunit consists of a large extracellular amino-terminal domain, four transmembrane domains, a cytoplasmic loop of variable length between the third and fourth transmembrane domains, and a short extracellular carboxy-terminal domain (Figure 1B) (Unwin 2005). The large extracellular domain of α subunits contain adjacent cysteines important for ligand binding whereas β subunits lack these residues (Albuquerque et al 2009). Unlike other α subunits, however, α5 does not contribute to ligand binding as it is missing a key tyrosine residue (Karlin 2002). Importantly though, incorporation of the α5 subunit into a mature receptor does alter receptor biophysical properties such as increasing the calcium conductance (Gerzanich et al 1998).

The combination of different nAChR subunits can lead to the formation of a vast array of nAChR subtypes. The α2 – α6 subunits can form heteromeric receptors with the β2 -β4 subunits while the α7, α8 and α9 subunits can form homomeric receptors that are blocked by α-bungarotoxin (Couturier et al 1990, Elgoyhen et al 1994, Schoepfer et al 1990). In addition, α9 can form a heteromeric receptor with α10 (Elgoyhen et al 2001, Lustig et al 2001) and α7 can form a heteromeric receptor with β2 (Liu et al 2009). Each of these receptor subtypes has distinct electrophysiological and pharmacological properties (Albuquerque et al 2009, Boyd 1997, Gerzanich et al 1997, Role and Berg 1996).

The functional diversity by the nAChR family offers abundant prospects for the design of novel therapeutics. As such, nAChRs are being actively investigated as drug targets for

nervous system disorders including Alzheimer's disease, anxiety, attention deficit hyperactivity disorder (ADHD), depression, epilepsy, pain, Parkinson's disease, schizophrenia, Tourette's syndrome, and nicotine addiction (Arneric et al 2007, Lloyd and Williams 2000, Romanelli et al 2007).

## The α3/α5/β4 nAChR subunit gene cluster

In recent years, a series of linkage analyses, candidate-gene association studies, and genome-wide association studies (GWAS) pointed to a possible role for the α3, α5, and β4 nAChR subunits in nicotine addiction as well as lung cancer (Amos et al 2008, Berrettini et al 2008, Bierut et al 2008, Caporaso et al 2009, Freathy et al 2009, Hung et al 2008, Pillai et al 2009, Portugal and Gould 2008, Saccone et al 2009a, Sasaki et al 2009, Schlaepfer et al 2007, Spitz et al 2008, Stevens et al 2008, Thorgeirsson et al 2008, Wacholder et al 2008, Weiss et al 2008). The genes that encode the α3, α5, and β4 nAChR subunits lie in a genomic cluster in strong linkage disequilibrium with each other (Figure 2) (Boulter et al 1990). These three genes encode a predominant nAChR subtype expressed in the peripheral nervous system (PNS) (Leonard and Bertrand 2001).

The function of the clustered subunits can be gleaned from knockout (KO) animal studies. These studies have shown that the α3 subunit is necessary for survival, with homozygous KO mice dying perinatally due to multiorgan dysfunction (Xu et al 1999b). α3 heterozygous (+/−) mice have enlarged bladders, causing bladder infection, dribbling urination, and urinary stones – a phenotype resembling that of a rare human condition called megacystis-microcolon-intestinal hypoperistalsis syndrome. Patients with this disease also do not appear to express α3 mRNA (Richardson et al 2001). α3 heterozygous mice also display extreme mydriasis and lack of pupil contraction in response to light, with loss of bladder contraction in response to nicotine (Xu et al 1999c). Furthermore, α3 heterozygous mice are partially resistant to nicotine-induced seizures compared to wild-type littermates (Salas et al 2004a). In contrast, α5 and β4 KO mice are both viable and lack any gross abnormalities (Wang et al 2002, Wang et al 2003, Xu et al 1999c). However, α5 and β4 KO mice do exhibit autonomic dysfunction and are less sensitive to nicotine. Mice lacking α5 are also more susceptible to experimentally induced inflammatory bowel disease (Orr-Urtreger et al 2005) while β4 KO mice display less anxiety in behavioral tests (Salas et al 2003).

The observation that the α3, α5, and β4 genes are co-expressed and co-regulated in many cell types in the nervous system is consistent with the hypothesis that their expression is due to coordinated transcriptional regulation. The three subunits are highly expressed in the PNS as well as in several regions of the brain including the brain stem, cerebellum, hippocampus, interpeduncular nucleus, medial habenula, pineal gland, and the ventral tegmental area (VTA) (Flora et al 2000b, Gahring et al 2004, Klink et al 2001, Perry et al 2002, Quick et al 1999, Salas et al 2003, Salminen et al 2004, Turner and Kellar 2005, Xu et al 1999b, Zoli et al 2002). Furthermore, mRNA levels of the three genes are coordinately up-regulated during neural development and differentiation (Corriveau and Berg 1993, Levey et al 1995, Levey and Jacob 1996, Zhou et al 1998).

Efforts have been made to understand the regulatory mechanisms governing the expression of the clustered nAChR subunit genes. Sequencing of the individual gene promoters has revealed that each promoter lacks classical CAAT and TATA boxes (Boulter et al 1990). Instead, the promoters are GC-rich and contain several binding sites for the transcription factor, Sp1. Sp1 regulates transcription of each of the clustered subunit genes through multiple binding sites in each individual promoter (Bigger et al 1997, Campos-Caro et al 1999, Campos-Caro et al 2001, Flora et al 2000a, Melnikova et al 2000, Melnikova and Gardner 2001, Terzano et al 2000, Valor et al 2002, Yang et al 1995). Chromatin

immunoprecipitation (ChIP) experiments have confirmed Sp1 binding in the context of native chromatin for all three promoters (Benfante et al 2007, Scofield et al 2008). It is likely that Sp1 is involved in tethering the basal transcription machinery to the TATA-less nAChR subunit gene promoters (Pugh and Tjian 1991). In addition to the Sp1 regulation common to all three promoters, other transcription factors have been found to govern expression of the clustered genes either independently or coordinately including ASCL1, Brn-3a-c, c-Jun, hnRNPK, PHOX2A, Purα, Sox10, Sp3, Tst-1/Oct6/SCIP (Benfante et al 2007, Bigger et al 1997, Du et al 1997, Du et al 1998, Improgo et al 2010, Liu et al 1999, Melnikova et al 2000, Milton et al 1996, Yang et al 1994). Two regulatory elements have also been found that direct the expression of the clustered nAChR genes in a tissue-specific manner: β43′, found at the β4 3′-untranslated region, and CNR4, a conserved noncoding region located 20 kb upstream of β4 (Xu et al 2006). Recently, we showed that a 2.3-kb fragment of the β4 gene promoter directs spatially and developmentally regulated expression of a reporter gene in vivo (Bruschweiler-Li et al 2010). Whether this region also regulates expression of the α3 and α5 genes remains to be determined.

## Role of nAChRs in nicotine addiction

Nicotine is one of the most widely consumed psychoactive drugs in the world and is the primary reinforcing chemical in tobacco (Stolerman and Jarvis 1995). Nicotine addiction is initiated upon nicotine-mediated activation of nAChRs in the mesolimbic dopaminergic (DAergic) pathway, known as the reward circuitry of the brain (Corrigall et al 1992, Dani and De Biasi 2001, Di Chiara 2000). DAergic neurons in this pathway originate in the VTA and project to the nucleus accumbens (NAc) and the prefrontal cortex. Activation of nAChRs expressed in the VTA ultimately causes an increase in the firing of DAergic neurons, resulting in an increase of DA release in the NAc (Calabresi et al 1989, Nisell et al 1994, Pidoplichko et al 1997, Pontieri et al 1996). Expression of α4- and β2-containing receptors in the VTA is necessary and sufficient for nicotine- mediated DA elevation in the NAc (Marubio et al 2003, Maskos et al 2005, Picciotto et al 1998, Pons et al 2008). α4β2* nAChRs are also critical for nicotine reward/reinforcement, sensitization, and tolerance (Picciotto et al 1998, Pons et al 2008, Tapper et al 2004, Tapper et al 2007). Elevation of DA levels in the NAc reinforces drug use and is critical for the onset and maintenance of nicotine dependence (Di Chiara and Imperato 1988). Conversely, inhibiting DA elevation via lesions or pharmacological blockade attenuates the rewarding effects of nicotine (Corrigall and Coen 1991).

Nicotine dependence is a consequence of both positive reinforcement as well as avoidance of the aversive effects of cessation (Kenny and Markou 2001). Smoking cessation produces withdrawal symptoms, which account for the high incidence of relapse in people attempting to quit smoking (Corrigall et al 1989, Kenny and Markou 2001). The withdrawal syndrome involves both mood-oriented (affective) as well as physical (somatic) symptoms (De Biasi and Salas 2008). α5- and β4-containing nAChRs as well as α7 nAChRs appear to be involved in the physical symptoms of withdrawal as somatic signs are diminished in α5, α7, and β4 KO mice (Jackson et al 2008, Salas et al 2004b, Salas et al 2007). Conversely, affective symptoms are absent in β2 KO mice but are readily observable in α5 and α7 KO mice (Jackson et al 2008, Portugal et al 2008).

Results of the aforementioned genetic studies also support the role of the α3, α5, and β4 subunits in nicotine dependence. In a candidate-gene study targeting 348 genes, smokers of European descent who developed nicotine dependence were compared to smokers who were not dependent (Saccone et al 2007). In this study, several SNPs associated with nicotine dependence were found within the α5/α3/β4 gene cluster. Of particular interest is the non-synonymous SNP, rs16969968, found in the fifth exon of the α5 gene. This polymorphism

changes an aspartic acid residue into asparagine at position 398 (D398N) in the second intracellular loop of α5. Receptors expressing the aspartic acid variant show greater maximal response to nicotine, causing higher intracellular calcium levels (Bierut et al 2008). Individuals with one copy of the minor allele were found to have a 1.3-fold increased risk for nicotine dependence while individuals with two copies of this risk variant have almost a 2-fold increase in risk (Saccone et al 2007). rs16969968 was also found to be associated with pleasurable responses during smoking initiation among Caucasians (Sherva et al 2008).

Other SNPs highly correlated with rs16969968 also influence the risk for nicotine dependence such as rs1051730 found in exon 5 of α3 and rs578776 found in the α3 3′-untranslated region (Saccone et al 2007). The latter had an even stronger association with nicotine dependence. These same SNPs were associated with increased smoking intake in an independent study analyzing 219 European American families (Bierut et al 2008). Furthermore, these SNPs were associated with early onset smoking, a phenotype associated with more severe nicotine dependence in adults (Weiss et al 2008). rs1051730 was also found to be strongly associated with smoking quantity in an Icelandic population (Thorgeirsson et al 2008) and was associated with decreased likelihood of quitting during pregnancy in women of European descent (Freathy et al 2009). These studies provide compelling evidence for the role of the α5/α3/β4 gene cluster in nicotine dependence.

## Role of nAChRs in lung cancer

Smoking is the major risk factor associated with lung cancer, the leading cause of cancer-related deaths for both men and women (ACS 2009). Lung cancer is also the second most common form of cancer in both sexes, with an overall five-year survival rate of 15%. The two major histopathological types of lung cancer are small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC can be subdivided into adenocarcinoma, squamous cell, bronchioalveolar, and large cell lung carcinoma. Greater than 95% of patients with SCLC have a history of cigarette smoking and five-year survival rates for these patients can reach as low as 2% (Jackman and Johnson 2005).

Several lines of evidence indicate that nAChRs play a role in lung carcinogenesis as discussed in the following sections. nAChRs are expressed in both normal and lung cancer cells (Improgo et al 2010, Lam et al 2007, Maneckjee and Minna 1990, Maus et al 1998, Sartelet et al 2008, Schuller 1989, Song et al 2003, Wang et al 2001). The clustered nAChR subunits, in particular, are over-expressed in SCLC (Improgo et al 2010). This over-expression appears to be regulated by achaete-scute complex homolog-1 (ASCL1)(Improgo et al 2010), a basic helix-loop-helix transcription factor that is also over-expressed in SCLC (Ball et al 1993). Transgenic mice that constitutively express ASCL1 and the SV40 large T antigen develop aggressive lung tumors with SCLC features (Linnoila et al 2000). Up-regulation of the clustered nAChRs by ASCL1 provides a mechanism by which the effects of nicotine and other nAChR ligands are potentiated in SCLC, contributing to the aggressiveness of this type of lung cancer (Improgo et al 2010). Additional evidence for a role of the clustered nAChR genes in lung cancer comes from the recent demonstration that the α3 subunit gene is a frequent target of aberrant DNA hypermethylation and silencing in lung cancer (Paliwal et al 2010).

## nAChRs and cell proliferation

The various ligands that activate nAChRs promote the development and progression of lung cancer via different mechanisms. First, ACh is synthesized by and acts as an autocrine growth factor for SCLC (Song et al 2003). ACh has also been shown to activate signaling pathways vital for growth and differentiation of human epithelial cells (Grando 2008). Similarly, nicotine can induce cell proliferation in a manner reminiscent of classical growth

factors activating cancer signaling pathways. Specifically, nicotine treatment has been shown to cause physical interactions between the retinoblastoma protein (Rb) and the signaling kinase Raf-1, leading to downstream events such as inactivation of cyclins and cyclin-dependent kinases, dissociation of the transcription factor E2F1 from Rb, binding of E2F1 to proliferative promoters causing their transcription, and entry into S-phase (Dasgupta and Chellappan 2006, Egleton et al 2008). In addition, nicotine treatment can increase the levels of growth factors such as brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), plateletderived growth factor (PDGF), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and vascular endothelial growth factor C (VEGF-C) as well as the corresponding growth factor receptors such as EGFR, HGFR, PDGFR, and VEGFR-2 (Conti-Fine et al 2000). Moreover, nicotine activation of EGFR appears to involve increases in intracellular calcium levels (Sher et al 1998). Nicotine also stimulates NSCLC cell proliferation by up-regulating fibronectin expression while down-regulating epithelial markers such as E-cadherin and β-catenin (Davis et al 2009, Zheng et al 2007b). Nicotine-induced fibronectin expression is associated with activation of the extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase (PI3-K)/mammalian target of rapamycin (mTOR) signaling pathways and is abrogated by treatment with the α7 nAChR antagonist, α-bungarotoxin (Zheng et al 2007a). This group also showed that nicotine induces NSCLC cell proliferation by stimulating the expression of the nuclear hormone receptor, peroxisome proliferator- activated receptor β/δ (PPARβ/δ), an effect that can be blocked by α-bungarotoxin, α7 nAChR short interfering RNA (siRNA), and PI3-K inhibitors (Sun et al 2009). Taken together, these results suggest that nicotine increases PPARβ/ gene expression through α7 nAChR–mediated activation of PI3K/mTOR signals leading to cell proliferation (Sun et al 2009, Zheng et al 2007a). Nicotine also promotes cell proliferation in other types of cancers: Nicotine promotes growth of gastric tumors by activating ERK and cyclooxygenase-2 and promotes growth of colon cancer via EGFR, c-Src, and 5-lipooxygenase-mediated signaling pathways (Shin et al 2004, Ye et al 2004).

## nAChRs and apoptosis

John Minna's group first showed that low concentrations of nicotine confer resistance to apoptosis in lung cancer cells (Maneckjee and Minna 1994). Since then, nicotine has been shown to inhibit apoptosis induced by various stress stimuli including UV radiation, oxidative stress, and exposure to opioids, $Ca^{2+}$ ionophores, neurotoxins, and anticancer drugs (Egleton et al 2008, Zeidler et al 2007). This apoptotic inhibition appears to involve several signaling pathways. One mechanism involves phosphorylation and consequent activation of the anti-apoptotic protein, B cell lymphoma gene 2 (BCL2) by protein kinase Cα and phospholipase C (Mai et al 2003). Consistently, nicotine inactivates the pro-apoptotic functions of Bax and Bad (Jin et al 2004, Xin and Deng 2005). Another mechanism involves nicotine-mediated activation of Akt (also called protein kinase B), a serine-threonine kinase whose activation leads to apoptotic inhibition and tumorigenesis (Scheid and Woodgett 2001). Nicotine exposure causes site-specific phosphorylation of Akt at Thr308 and Ser473 as well as phosphorylation of downstream Akt substrates such as mTOR, FKHR, elf-4, GSK3B, tuberin, and S6K (West et al 2003). The use of pharmacological agents suggests that this process involves α3-containing nAChRs. In the same study, increased Akt activation was observed in lung cancer tissue from smokers. Further evidence implicating α3 in Akt signal transduction is a recent report demonstrating that small hairpin RNA-mediated depletion of the α3 subunit leads to a dramatic $Ca^{2+}$ influx in a NSCLC cell line that was followed by activation of the Akt pathway (Paliwal et al 2010). In this study, NSCLC cells in which the α3 subunit was depleted were resistant to apoptosis-inducing drugs.

## nAChRs and angiogenesis

Endothelial cells express nAChRs as well as key molecules for cholinergic signaling such as choline acetyltransferase and acetylcholinesterase (Macklin et al 1998, Wang et al 2001). In these cells, ACh is thought to act in an autocrine or paracrine manner to stimulate angiogenesis (Cooke and Ghebremariam 2008). Nicotine also functions as a pro-angiogenic agent, activating both physiologic and pathologic angiogenesis via the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (Heeschen et al 2001). Analogous to angiogenic cytokines, nicotine promotes endothelial cell migration, proliferation, survival, tube formation, and nitric oxide production and can be as potent as fibroblast growth factor (Cooke and Ghebremariam 2008). Nicotine and its metabolite, cotinine, have also been shown to up-regulate the expression of VEGF in endothelial cells (Conklin et al 2002). In addition, second-hand smoke increases VEGF expression and elevates levels of circulating endothelial progenitor cells, promoting angiogenesis and tumor growth - an effect reduced by the non-selective nAChR antagonist mecamylamine (Zhu et al 2003). Even in the absence of exogenous nicotine, angiogenic processes stimulated by VEGF or FGF can be blocked by nAChR antagonists such as mecamylamine and hexamethonium and the α7-selective antagonist α-bungarotoxin (Cooke and Ghebremariam 2008). In lung cancer cells, nicotine also induces the expression of hypoxia-inducible factor-1 alpha (HIF-1α), a transcription factor that promotes hypoxia-induced angiogenesis (Zhang et al 2007).

## nAChRs and the immune system

The function of nAChRs in immunity and cancer has two aspects. The first involves the complex interplay between the inflammatory effects of irritants in cigarette smoke and the anti-inflammatory effects of nicotine (Gahring and Rogers 2006). Chronic inflammation triggered by tobacco smoke has been shown to promote lung carcinogenesis (Takahashi et al 2010). Inflammation induced by cigarette smoke also promotes COPD, a disease associated with increased lung cancer risk (Grivennikov et al 2010, Punturieri et al 2009). Chronic inflammation increases cancer risk by influencing every stage of cancer from initiation, promotion, invasion, and metastasis via induction of oncogenic mutations and genomic instability, local immunosuppression, and angiogenesis (reviewed in (Grivennikov et al 2010). In contrast, nicotine itself appears to suppress immune function and has been shown to be protective against inflammatory diseases such as pneumonia and ulcerative colitis (Blanchet et al 2004, Rubin and Hanauer 2000, Shivji et al 2005). Suppression of the immune response by nicotine may impact immune surveillance, preventing the clearance of nascent tumor cells (Gahring and Rogers 2006, Grivennikov et al 2010).

The second aspect of nAChR function in immunity and cancer involves the production of autoantibodies against nAChRs in cancer patients with paraneoplastic syndromes (Gahring and Rogers 2006). In particular, antibodies against α3 nAChRs have been detected in the serum of SCLC patients that display autonomic neuropathy (Vernino et al 1998, Vernino et al 2000). Dysautonomia caused by these autoantibodies is characterized by symptoms such as impaired papillary light reflex, gastrointestinal dysmotility, and bladder dysfunction that are reminiscent of those observed in α3 heterozygous KO mice (McKeon et al 2009, Xu et al 1999a).

## Carcinogenic nitrosamines as nAChR ligands

Nicotine-derived nitrosamines such as NNK and NNN activate nAChRs with varying affinities (Schuller and Orloff 1998). NNK preferentially activates α7 nAChRs while NNN has higher affinity for heteromeric nAChRs. Activation of nAChRs by these ligands promotes cell proliferation, apoptotic inhibition, and angiogenesis (Schuller 2009). NNK

and NNN appear to stimulate distinct proliferative pathways in bronchial epithelial cells. NNK causes activation of the transcription factors GATA-3, NF-KB, and STAT-1 while NNN predominantly activates GATA-3 and STAT-1, effects that can be abolished by the nAChR antagonists α-bungarotoxin and mecamylamine, respectively (Arredondo et al 2006a). In SCLC cells, NNK promotes calcium influx, serotonin release, and activation of the PKC and Raf-1/MAPK pathway (Arredondo et al 2006b, Jull et al 2001, Schuller 1992). NNK has also been shown to activate the Akt pathway *in vitro* and inhibit apoptosis (West et al 2003). In the same study, increased Akt phosphorylation was found in the lungs of NNK-treated mice. These studies suggest that carcinogenic nitrosamines can initiate lung cancer via their genotoxic effects but can also promote lung cancer via nAChR-mediated mechanisms (Arredondo et al 2006a).

## Risk alleles in lung cancer

Several SNPs found in the α5/α3/β4 gene cluster appear to influence the risk for lung cancer. In a large-scale GWAS involving approximately 317,000 SNPs in samples of European origin, the non-synonymous SNP, rs16969968, was found to be strongly associated with lung cancer (Hung et al 2008). This SNP was also found to increase the risk for lung adenocarcinoma in an Italian population (Falvella et al 2009). Hung and colleagues also showed that the increased risk for lung cancer was observed even in non-smokers, suggesting that the association is not simply a consequence of nicotine dependence. Another evidence for direct association is that rs16969968 did not increase the risk for other smoking-related cancers such as head and neck cancer.

The α3 exon 5 SNP, rs1051730, was also found to be associated with lung cancer (Hung et al 2008). Furthermore, in an independent GWAS, rs1051730 was found to be associated with lung cancer and was only weakly associated with nicotine dependence (Amos et al 2008). rs1051730 was also found to be associated with familial lung cancer even after adjustment for pack-years of cigarette exposure (Liu et al 2008). Another group also found rs1051730 to be associated with lung cancer and peripheral arterial disease (Thorgeirsson et al 2008). Taken together, these studies represent a strong convergence of genetic data implicating the α3/α5/β4 gene cluster in lung cancer.

One report, however, showed that the rs1051730 SNP was associated with both nicotine dependence and lung cancer but that there was no increased risk for lung cancer in lifetime never-smokers, suggesting that the association with lung cancer was an effect of nicotine dependence (Thorgeirsson et al 2008). Reasons for the conflicting data may include differences in populations, sample sizes, phenotypes used to assess nicotine dependence and instruments used to measure phenotypes (Greenbaum and Lerer 2009). For example, most of the studies were done in populations of European origins where the frequency of the rs16969968 allele is 37% whereas in African populations the frequency of this allele is significantly lower (Bierut et al 2008, Saccone et al 2009b).

## Conclusions and perspectives

Given the number of carcinogens found in cigarettes, it is not surprising that smoking is the major risk factor associated with lung cancer. Hence, many mechanisms leading to cancer can be envisaged. One such mechanism involves the activation of nAChRs by nicotine and its metabolites, which subsequently engage cancer signaling pathways associated with cell proliferation, apoptotic inhibition and angiogenesis. Previous studies investigating the link between nAChRs and these pathways have implicated primarily the α7 nAChR. The recent deluge of genetic studies, however, suggests that other subtypes should be investigated, in particular, the α3/α5/β4 nAChR subtype. Our work demonstrating the over-expression of the

clustered nAChR genes in SCLC and their regulation by ASCL1, a critical player in the pathogenesis of lung cancer, provides evidence for the role of the clustered nAChR genes in this disease (Improgo et al 2010). This is further substantiated by the recent finding of aberrant DNA hypermethylation and silencing of the α3 subunit gene in NSCLC (Paliwal et al 2010). The use of genetic approaches to investigate the non-synonymous SNP found in α5 as well as other SNPs found in the cluster should be fertile areas for future investigations.

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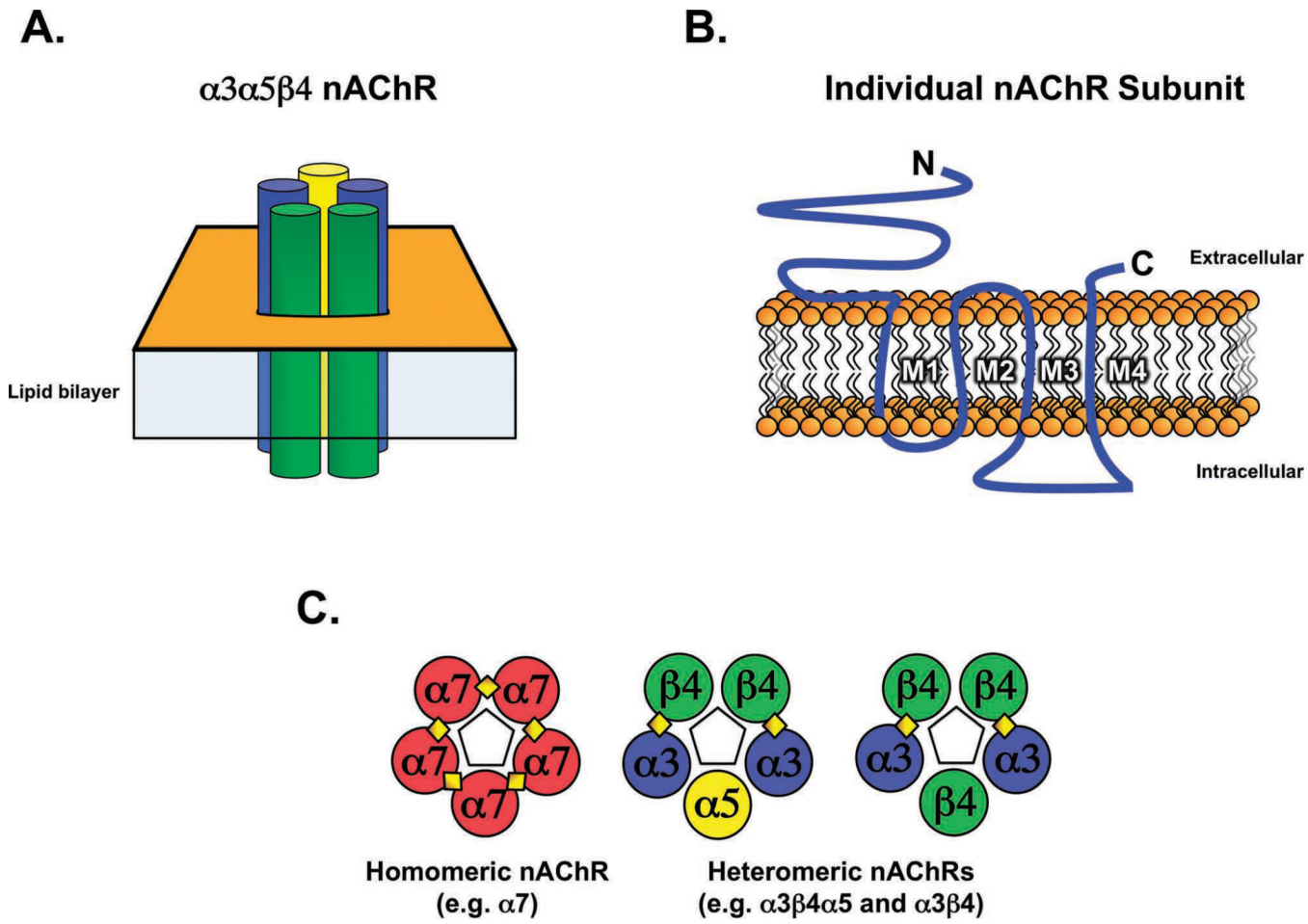



**Figure 1. Structure of the nAChR**

A.) Schematic representation illustrating the pentameric arrangement of subunits in an assembled nAChR. B.) Conserved domains of a nAChR subunit including the amino (N) and carboxy (C) terminals, transmembrane segments (M1–M4), and the intracellular loop. C.) Assembly of heteromeric and homomeric nAChR subtypes. Individual nAChR subunits are represented as colored circles, with diamonds representing ligand-binding sites. Pentagons in the center of each pentamer represent the pore region.

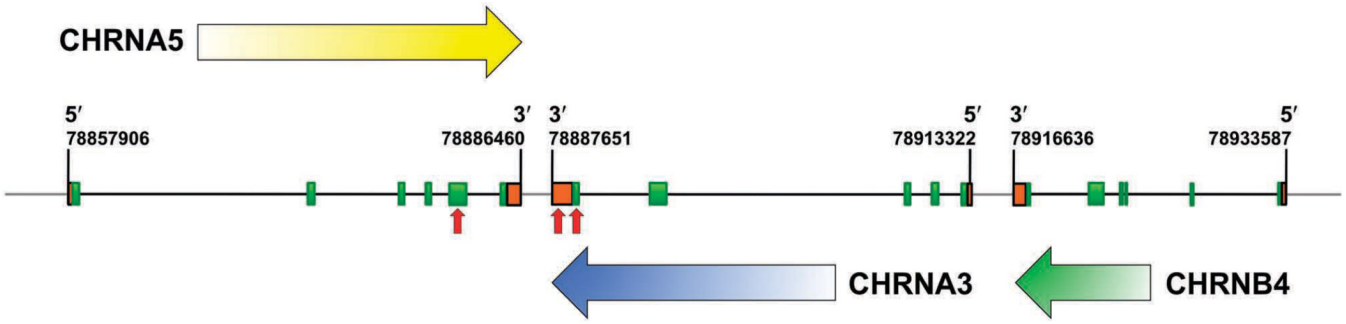
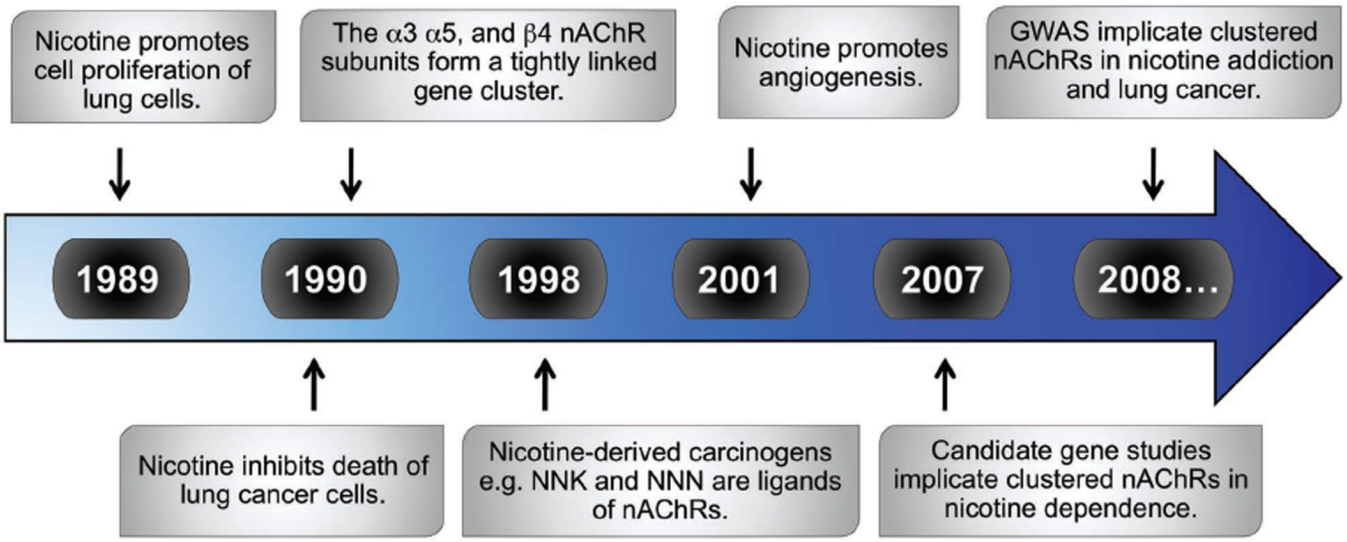



**Figure 2. The human nAChR α3/α5/β4 gene cluster**

Green boxes represent exons and oragne boxes represent untranslated regions. Black lines located between green boxes represent introns while gray lines represent intragenic regions. The boundaries for each gene are labeled with corresponding Genbank annotations. Horizontal arrows indicate the direction of transcription. Vertical red arrows indicate SNPs associated with nicotine dependence and lung cancer.




**Timeline. Key events implicating nAChRs in lung cancer etiology**

nAChR, nicotinic acetylcholine receptor; NNK [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone]; NNN, [N-nitrosonornicotine] GWAS, genome wide association study.