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The nicotinic acetylcholine receptor CHRNA5/A3/B4 gene cluster: Dual role in nicotine addiction and lung cancer

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Abstract

More than 1 billion people around the world smoke, with 10 million cigarettes sold every minute. Cigarettes contain thousands of harmful chemicals including the psychoactive compound, nicotine. Nicotine addiction is initiated by the binding of nicotine to nicotinic acetylcholine receptors, ligand-gated cation channels activated by the endogenous neurotransmitter, acetylcholine. These receptors serve as prototypes for all ligand-gated ion channels and have been extensively studied in an attempt to elucidate their role in nicotine addiction. Many of these studies have focused on heteromeric nicotinic acetylcholine receptors containing $\alpha 4$ and $\beta 2$ subunits and homomeric nicotinic acetylcholine receptors containing the $\alpha 7$ subunit, two of the most abundant subtypes expressed in the brain. Recently however, a series of linkage analyses, candidate-gene analyses and genome-wide association studies have brought attention to three other members of the nicotinic acetylcholine receptor family: the $\alpha 5$, $\alpha 3$ and $\beta 4$ subunits. The genes encoding these subunits lie in a genomic cluster that contains variants associated with increased risk for several diseases including nicotine dependence and lung cancer. The underlying mechanisms for these associations have not yet been elucidated but decades of research on the nicotinic receptor gene family as well as emerging data provide insight on how these receptors may function in pathological states. Here, we review this body of work, focusing on the clustered nicotinic acetylcholine receptor genes and evaluating their role in nicotine addiction and lung cancer.

Keywords

Nicotinic acetylcholine receptors; CHRNA5/A3/B4 gene cluster; Nicotine addiction; Lung cancer; Genome-wide association studies

1. Introduction

The molecular cloning of nicotinic acetylcholine receptors (nAChRs) from brain cDNA libraries in the mid-1980s was a watershed event as it opened the window to not only a molecular understanding of cholinergic signaling within the nervous system, but also to a structural understanding of how members of the Cys-loop superfamily of ligand-gated ion channels function. In addition to neuronal and muscle nAChRs, members of this family include the ionotropic receptors for glycine, 5-hydroxytryptamine (5-HT₃), and γ -aminobutyric acid (GABA_A and GABA_C) (Dani and Bertrand, 2007; Le Novère and Changeux, 1995). As the

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cloning frenzy subsided, a tremendous amount of effort was put forth to understand the pharmacological and biophysical diversity of nAChRs. As a result, nAChRs are among the most well understood allosteric membrane proteins from a structural and functional point of view (Albuquerque *et al.*, 2009). Furthermore, nAChRs have emerged as key therapeutic targets for a variety of pathologies including schizophrenia, depression, attention deficit hyperactivity disorder, Alzheimer's disease and of course, tobacco addiction (Arneric *et al.*, 2007; Levin and Rezvani, 2007; Romanelli *et al.*, 2007; Taly *et al.*, 2009). More recently, genetic studies have identified single nucleotide polymorphisms (SNPs) in the chromosomal locus encoding three nAChR genes as risk factors for 1) nicotine dependence 2) lung cancer, 3) chronic obstructive pulmonary disease, 4) alcoholism and 5) peripheral arterial disease (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; Saccone *et al.*, 2009b; Saccone *et al.*, 2007; Sasaki *et al.*, 2009; Schlaepfer *et al.*, 2008; Stevens *et al.*, 2008; Thorgeirsson *et al.*, 2008; Weiss *et al.*, 2008). This cluster of nAChR genes encodes the $\alpha 3$, $\alpha 5$ and $\beta 4$ subunits (designated CHRNA5/A3/B4) (Boulter *et al.*, 1990). As a result of these genetic studies, new attention has been brought to bear on the CHRNA5/A3/B4 genes and their encoded subunits. This review summarizes recent work on the clustered nAChR subunits highlighting their structure, function, and expression in a variety of pathological conditions.

2. Neuronal nicotinic acetylcholine receptors

2.1 General overview

Signaling through neuronal nAChRs underlies several fundamental biological processes both during development and in the adult (Albuquerque *et al.*, 2009). In the central nervous system (CNS), presynaptic nAChRs modulate release of most classical neurotransmitters including norepinephrine, acetylcholine (ACh), glutamate and GABA (McGehee *et al.*, 1995). Postsynaptic nAChRs are intimately involved in fast ACh-mediated synaptic transmission in addition to activity-dependent gene expression, which is critical for synaptic plasticity (Albuquerque *et al.*, 2009; Dani and Bertrand, 2007; Hu *et al.*, 2002; Ji *et al.*, 2001). Within the peripheral nervous system (PNS), nAChRs mediate fast excitatory transmission in most, if not all, autonomic ganglia and are involved in modulating visceral and somatic sensory transmission (Boyd *et al.*, 1991; Genzen *et al.*, 2001; Hu and Li, 1997; Steen and Reeh, 1993; Sucher *et al.*, 1990; Wang *et al.*, 2002b). More recently, numerous studies have revealed the expression of nAChRs on non-neuronal cells such as lung, glia, keratinocytes, endothelial cells, as well as cells of the digestive and immune systems, and evidence is accumulating indicating that the receptors play crucial roles in signal transduction underlying many physiological processes outside the nervous system (Arredondo *et al.*, 2001; Battaglioli *et al.*, 1998; Gahring *et al.*, 2004b; 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). The importance of nAChR-mediated signaling is reflected in the many pathologies in which cholinergic signal transduction is compromised. For example, significant alterations in nAChR expression and function have been documented in several diseases such as Alzheimer's disease, autosomal dominant nocturnal frontal lobe epilepsy, megacystis-microcolon-intestinal hypoperistalsis syndrome, Parkinson's disease, schizophrenia, and Tourette's disease (De Fusco *et al.*, 2000; Isacson *et al.*, 2002; Lena and Changeux, 1997; Perl *et al.*, 2003; Perry *et al.*, 2001; Quik, 2004; Quik *et al.*, 2007; Richardson *et al.*, 2001; Silver *et al.*, 2001; Steinlein *et al.*, 1995; Teaktong *et al.*, 2003; Whitehouse *et al.*, 1988; Zanardi *et al.*, 2002). In addition, nAChRs are key players in the initial steps and subsequent downstream health consequences of nicotine addiction (Kedmi *et al.*, 2004; Laviolette and van der Kooy, 2004). Coupled to this is a growing awareness that nAChRs may directly contribute to the pathogenesis of lung cancer (Catassi *et al.*, 2008; Eggleton *et al.*, 2008; Schuller, 2008, 2009; Song *et al.*, 2008).

2.2. Structural and functional properties of nAChRs

Neuronal nAChRs are transmembrane proteins that form pentameric structures assembled from a family of subunits that include $\alpha 2$ – $\alpha 10$ and $\beta 2$ – $\beta 4$ (Brejc *et al.*, 2001; Cooper *et al.*, 1991). Each nAChR subunit consists of an approximately 200-residue extracellular N-terminus, four transmembrane segments (designated M1–M4), a variable intracellular loop (100–200 residues) between M3 and M4, and an extracellular C-terminus (4–28-residue, Fig. 1A) (Corringer *et al.*, 2000). The N-terminus contains the ACh-binding domain (Eisele *et al.*, 1993) with the interface between adjacent subunits forming a hydrophobic pocket that serves as the agonist-binding site. The M2 transmembrane segment of all 5 subunits forms the conducting pore of the channel, with regions in the M1–M2 intracellular loop contributing to cation permeability and agonist binding affinities (Corringer *et al.*, 2000; McGehee and Role, 1995). When activated by an agonist in native or heterologous expression systems, nAChRs exhibit a rapid (500 ms to peak current, $[ACh] = EC_{50}$) inward current that desensitizes (Barnard *et al.*, 1982) and is potentiated by calcium ions (Vernino *et al.*, 1992). The functional diversity exhibited by the neuronal nAChR family is a consequence, in large part, of the differential expression of the various subunit genes leading to the incorporation of distinct subunits into mature receptors.

Much of what is known about the biophysical and pharmacological properties of nAChRs is based on studies in heterologous expression systems (McGehee and Role, 1995). These systems make use of nAChR mRNA or cRNA injected into *Xenopus* oocytes as well as nAChR cDNA transfected into mammalian cell lines in order to express nAChR subunits singly or in combination. The propensity for nAChR subunits to form homomeric subtypes was determined by expressing subunits singly. When expressed alone, $\alpha 7$, $\alpha 8$, $\alpha 9$, and $\alpha 10$ are able to form functional receptors (Couturier *et al.*, 1990a; Gerzanich *et al.*, 1994). In contrast, other α subunits require the presence of β subunits to form functional receptors (Fig. 1B).

Of the clustered receptor subunit genes, co-expression of $\alpha 3$ and $\beta 4$ nicotinic receptor subunit cRNA results in functional receptors with a single channel conductance, γ , of ~24 pS (McGehee and Role, 1995; Nelson and Lindstrom, 1999). ACh and nicotine are less potent agonists of $\alpha 3\beta 4$ nAChRs compared to the prototypic high affinity $\alpha 4\beta 2$ nAChRs (Table 2). In addition, $\alpha 3$ can coassemble with $\beta 2$ nAChR subunits and form a functional receptor (Table 2) (Boulter *et al.*, 1987; Wada *et al.*, 1988). Unlike $\alpha 3$, the $\alpha 5$ subunit fails to form functional receptors when co-expressed with either $\beta 4$ or $\beta 2$ subunits (Boulter *et al.*, 1987; Couturier *et al.*, 1990b). Although the $\alpha 5$ subunit harbors the characteristic viscinal cysteines in the large extracellular domain, a tyrosine residue (Tyr198) required for ligand binding has been substituted with an aspartic acid residue, likely explaining the lack of functionality of this subunit when expressed with either β subunit alone (Albuquerque *et al.*, 2009; Karlin, 2002). Thus, for several years after its initial cloning, the impact of the $\alpha 5$ subunit on nAChR function was poorly understood. This mystery was solved when $\alpha 5$ was co-expressed with both $\alpha 4$ and $\beta 2$ subunits (Ramirez-Latorre *et al.*, 1996). ACh-evoked currents from *Xenopus* oocytes expressing the three subunits were distinct from ACh-evoked currents in oocytes expressing only $\alpha 4$ and $\beta 2$ subunits. In particular, expression of $\alpha 5$ dramatically shifted the ACh concentration response curve to the right and significantly increased the predominant single channel conductance ($\alpha 4\beta 2 = 24$ pS, $\alpha 4\alpha 5\beta 2 = 44$ pS at -100 mV). Finally, cysteine mutagenesis of amino acid residues in the M2 domain of the $\alpha 5$ nAChR subunit conferred MTSET sensitivity to $\alpha 4\beta 2^*$ nAChRs indicating participation of the $\alpha 5$ subunit M2 domain with the pore of assembled receptors (“*” denotes containing, (Lukas *et al.*, 1999). Later studies analyzed the impact of $\alpha 5$ expression on $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChR function. In contrast to its effects on the pharmacology of $\alpha 4\beta 2$ nAChRs, $\alpha 5$ expression increased the sensitivity of $\alpha 3\beta 2$ nAChRs to nicotine and ACh (Table 2) (Wang *et al.*, 1996). Surprisingly, the ACh/nicotine concentration-response relationship between $\alpha 3\beta 4$ and $\alpha 3\alpha 5\beta 4$ nAChRs was found to

not dramatically differ (Wang *et al.*, 1996). However, $\alpha 5$ subunit expression did accelerate the rate of desensitization of these receptors. In addition, expression of the $\alpha 5$ subunit reduced channel sensitivity to agonist in $\alpha 3\beta 4$ nAChRs with engineered point mutations in the M2 domain that renders receptors hypersensitive to agonist definitively illustrating that $\alpha 5$ nAChR subunits can co-assemble with $\alpha 3\beta 4$ nAChRs (Groot-Kormelink *et al.*, 2001). Finally, coassembly of the $\alpha 5$ subunit with $\alpha 3\beta 2$ or $\alpha 3\beta 4$ nAChRs increases the calcium permeability of the resulting receptors (Gerzanich *et al.*, 1998) indicating that these receptors could play significant roles in the initiation of ACh-induced signaling cascades under normal and pathological condition. In addition to uncovering a functional role of the $\alpha 5$ nAChR subunit, these studies demonstrated that a portion of functional nAChRs in native tissue likely consist of heteropentamers containing three or more distinct subunits.

2.3 The CHRNA5/A3/B4 gene cluster

The CHRNA5/A3/B4 nAChR subunit genes are found in a tight cluster in chromosomal region 15q24–25 (Fig. 1C) (Boulter *et al.*, 1990). Admixtures of the nAChR subunits encoded by this locus form the predominant nicotinic receptor subtypes expressed in the PNS (Conroy and Berg, 1995; Covernton *et al.*, 1994; Flores *et al.*, 1996; Rust *et al.*, 1994; Vernallis *et al.*, 1993) as well as at key sites in the CNS such as the medial-habenula (Gotti *et al.*, 2007; Grady *et al.*, 2009). To determine the function of the clustered nAChR subunits, knockout (KO) mice have been generated. Mice that do not express the $\alpha 3$ subunit usually die within a week of birth due to multi-organ dysfunction (Xu *et al.*, 1999a). $\alpha 3$ KO mice develop 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 (Xu *et al.*, 1999a). Consistently, patients with this disease do not appear to express $\alpha 3$ mRNA (Richardson *et al.*, 2001). $\alpha 3$ KO mice also display extreme pupil dilation and lack of pupil contraction in response to light and have retinal wave activity with altered spatiotemporal properties delaying the refinement of retinal ganglion cell dendrites (Bansal *et al.*, 2000; Xu *et al.*, 1999a). Bladder contraction in response to nicotine is also lost. In addition, electrophysiological characterization shows that nicotine-induced whole-cell currents are abolished in the superior cervical ganglion (SCG) of $\alpha 3$ KO mice.

In contrast to the $\alpha 3$ KO mice, $\alpha 5$ and $\beta 4$ KO mice are both viable and lack any gross abnormalities (Wang *et al.*, 2003; Wang *et al.*, 2002a; Xu *et al.*, 1999a). $\alpha 5$ KO mice do exhibit abnormal cardiac parasympathetic ganglionic transmission and are less sensitive to acute nicotine treatment. Loss of $\alpha 5$ selectively affects axonal nAChRs in the SCG while leaving somatodendritic receptors unaffected (Fischer *et al.*, 2005). Similarly, ganglionic transmission is impaired in $\beta 4$ KO mice, attenuating ileal and bladder contractile responses to nicotinic agonists. Nicotine-induced whole cell currents in the SCG of $\beta 4$ KO mice are also reduced but still present, suggesting that compensation from another subunit (i.e. $\beta 2$) may be occurring (Xu *et al.*, 1999b). Consistent with this notion, nicotine-induced currents in the SCG are abolished in double $\beta 2$ - $\beta 4$ KO mice. Moreover, double $\beta 2$ - $\beta 4$ KO mice exhibit similar bladder and pupil dysfunction as $\alpha 3$ KO mice. Taken together, these studies indicate that the clustered nAChR subunits are essential for normal ganglionic function and that compensation by $\beta 2$ can occur with the loss of $\beta 4$.

In addition to their PNS-specific phenotypes, $\alpha 3$, $\alpha 5$ and $\beta 4$ KO mice also exhibit CNS-centric abnormalities compared to WT mice. For example, $\alpha 3$, $\alpha 5$ and $\beta 4$ KO animals are resistant to nicotine-induced seizures compared to their respective WT littermates and are not as sensitive to nicotine-induced inhibition of locomotion (Salas *et al.*, 2004a; Salas *et al.*, 2003a). $\beta 4$ KO mice also appear less anxious compared to WT mice in two specific anxiety assays suggesting a role for $\beta 4^*$ nAChRs in modulating anxiogenic stimuli (Salas *et al.*, 2003b). These mice also

have a lower core body temperature which is less responsive to modulation by acute nicotine infusion (Sack *et al.*, 2005).

3. Regulation of CHRNA5/A3/B4 expression

3.1 Expression patterns of the CHRNA5/A3/B4 genes

Although the initial focus of nAChR expression was in the nervous system, it is now clear that “neuronal” nAChR genes are also expressed in non-neuronal cells where they participate in a number of fundamental processes (Gahring and Rogers, 2006; Sharma and Vijayaraghavan, 2002; Spindel, 2003; Wessler and Kirkpatrick, 2008). This is certainly true for the CHRNA5/A3/B4 subunit genes. The CHRNA5/A3/B4 genes are co-expressed in many cell types, thus their clustering may reflect coordinate regulation (Table 1). This hypothesis is supported by the fact that the transcriptional activities of the promoter regions of the three genes are regulated by many of the same transcription factors (Fig. 2). However, the CHRNA5/A3/B4 genes are not always co-expressed, suggesting that independent regulation of each gene also occurs (Table 1 and Fig. 2.).

3.1.1 CHRNA3—In the nervous system, the $\alpha 3$ subunit is highly expressed in the periphery with a more restricted expression profile in the CNS (Gotti and Clementi, 2004; Greenbaum and Lerer, 2009). In the PNS, $\alpha 3$ subunit expression is seen in trigeminal sensory neurons (Flores *et al.*, 1996; Liu *et al.*, 1998), facial motoneurons (Senba *et al.*, 1990), retina (Feller, 2002; Moretti *et al.*, 2004), dorsal root ganglia (Zoli *et al.*, 1995) as well as SCG, adrenal medulla, sphenopalatine and otic ganglia (Rust *et al.*, 1994). Centrally, the $\alpha 3$ subunit is expressed in the brainstem (Morley, 1997), cerebellum (Hellström-Lindhahl *et al.*, 1999; Turner and Kellar, 2005), spinal cord (Hellström-Lindhahl *et al.*, 1998; Keiger *et al.*, 2003), substantia nigra (Azam *et al.*, 2002), medial habenula (Grady *et al.*, 2009; Zoli *et al.*, 1995), pineal gland (Zoli *et al.*, 1995), hippocampus (Gahring *et al.*, 2004a; Guan *et al.*, 2002; Hellström-Lindhahl *et al.*, 1999; Terzano *et al.*, 1998; Winzer-Serhan and Leslie, 1997), cortex (Guan *et al.*, 2002; Hellström-Lindhahl *et al.*, 1999) thalamus (Perry *et al.*, 2002; Terzano *et al.*, 1998), ventral tegmental area (Greenbaum and Lerer, 2009; Perry *et al.*, 2002) and interpeduncular nucleus (Grady *et al.*, 2009; Perry *et al.*, 2002; Winzer-Serhan and Leslie, 1997).

Outside the nervous system, the $\alpha 3$ subunit is expressed in human oral keratinocytes (Arredondo *et al.*, 2005; Arredondo *et al.*, 2001; Conti-Tronconi *et al.*, 1994) where its expression, both mRNA and protein, is increased following exposure to nicotine (Arredondo *et al.*, 2005; Arredondo *et al.*, 2001; Zia *et al.*, 2000). $\alpha 3$ -containing nAChRs are also expressed in lymphocytes (Benhammou *et al.*, 2000), the gastrointestinal tract (Flora *et al.*, 2000a; Glushakov *et al.*, 2004), vascular endothelial cells (Macklin *et al.*, 1998; Wang *et al.*, 2001), polymorphonuclear cells (Benhammou *et al.*, 2000), bronchial epithelium (Maus *et al.*, 1998; Wang *et al.*, 2001) and O2A progenitors (Rogers *et al.*, 2001). The $\alpha 3$ subunit is also expressed in lung (Improgo *et al.*, 2010; Lam *et al.*, 2007; Sartelet *et al.*, 2008) and its expression increases in small cell lung carcinoma (Improgo *et al.*, 2010).

3.1.2 CHRNA5—Similar to the $\alpha 3$ subunit, the $\alpha 5$ subunit is most highly expressed in the PNS but is also expressed in several key regions of the CNS (Gotti and Clementi, 2004; Greenbaum and Lerer, 2009). Centrally, $\alpha 5$ is expressed primarily in the cerebellum and thalamus (Flora *et al.*, 2000a) but is also detected in the cortex, hippocampus, brainstem, spinal cord, habenula, interpeduncular nucleus and other midbrain nuclei (Azam *et al.*, 2002; Gahring *et al.*, 2004a; Grady *et al.*, 2009; Hellström-Lindhahl *et al.*, 1998; Keiger *et al.*, 2003; Wada *et al.*, 1990; Zoli *et al.*, 2002). In the PNS, $\alpha 5$ is expressed in most autonomic ganglia (Flora *et al.*, 2000a; Liu *et al.*, 1998) and the retina (Moretti *et al.*, 2004).

In addition to the nervous system, $\alpha 5$ subunit expression has been detected in the gastrointestinal tract, where its expression is substantially higher than that of the $\alpha 3$ subunit, thymus and testis (Flora *et al.*, 2000a; Glushakov *et al.*, 2004). Furthermore, the $\alpha 5$ subunit is expressed in many of the same cell types as the $\alpha 3$ and $\beta 4$ subunits including oral epithelium (Arredondo *et al.*, 2005; Arredondo *et al.*, 2001), vascular endothelial cells (Macklin *et al.*, 1998; Wang *et al.*, 2001), bronchial epithelium (Maus *et al.*, 1998; Wang *et al.*, 2001), O2A progenitors (Rogers *et al.*, 2001) and a variety of immune cells (Wessler and Kirkpatrick, 2008).

3.1.3 CHRNB4—As with the other two genes in the nAChR cluster, the $\beta 4$ subunit gene is widely expressed in the PNS with more limited expression centrally (Gotti and Clementi, 2004). $\beta 4$ expression is relatively high in trigeminal sensory neurons (Flores *et al.*, 1996; Liu *et al.*, 1998) as well as the superior cervical, dorsal root, sphenopalatine and otic ganglia and sympathetic neurons (Mandelzys *et al.*, 1994; Rust *et al.*, 1994; Zoli *et al.*, 1995). The $\beta 4$ subunit is also expressed in the adrenal medulla (Di Angelantonio *et al.*, 2003) with lower expression in the retina (Moretti *et al.*, 2004). In the CNS, $\beta 4$ expression is particularly high in the olfactory bulb, pineal gland, medial habenula and interpeduncular nucleus (Dineley-Miller and Patrick, 1992; Grady *et al.*, 2009; Winzer-Serhan and Leslie, 1997) with lower expression in other thalamic nuclei, the cortex, hippocampus, spinal cord, cerebellum and midbrain (Azam *et al.*, 2002; Gahring *et al.*, 2004a; Hellström-Lindahl *et al.*, 1998; Keiger *et al.*, 2003; Perry *et al.*, 2002; Quik *et al.*, 2000; Turner and Kellar, 2005).

Outside the nervous system, there is again significant overlap of $\beta 4$ expression with $\alpha 3$ and $\alpha 5$ expression. $\beta 4$ is expressed in multiple cell types of the intestine (Flora *et al.*, 2000a; Glushakov *et al.*, 2004), vascular endothelial cells (Macklin *et al.*, 1998), oral keratinocytes (Arredondo *et al.*, 2005; Arredondo *et al.*, 2001), polymorphonuclear cells (Benhammou *et al.*, 2000), bronchial epithelium (Maus *et al.*, 1998; Wang *et al.*, 2001) and O2A progenitors (Rogers *et al.*, 2001). Finally, $\beta 4$ is co-expressed with $\alpha 3$ and $\alpha 5$ in lung (Improgo *et al.*, 2010; Lam *et al.*, 2007; Sartelet *et al.*, 2008) and is also up-regulated in lung cancer (see below) (Improgo *et al.*, 2010).

3.2. Transcriptional regulation of the CHRNA3/A5/B4 genes

The co-expression of the CHRNA3/A5/B4 genes coupled with their genomic clustering suggests they may share common regulatory mechanisms in addition to specific regulation of each gene. Further support for this idea comes from several observations. First, nucleotide sequencing of the individual gene promoters revealed that they each lack classical CAAT and TATA boxes (Boulter *et al.*, 1990). Instead, the promoters are GC-rich and contain several binding sites for the transcription factors, Sp1 and Sp3 (Fig. 2). Both Sp factors positively regulate transcription of each of the clustered subunit genes through multiple binding sites in each individual promoter (Bigger *et al.*, 1996; Bigger *et al.*, 1997; Boyd, 1996; Campos-Caro *et al.*, 2001; Campos-Caro *et al.*, 1999; Flora *et al.*, 2000b; Melnikova and Gardner, 2001; Melnikova *et al.*, 2000a; Terzano *et al.*, 2000; Valor *et al.*, 2002; Yang *et al.*, 1995). Chromatin Immunoprecipitation (ChIP) experiments demonstrated Sp1 binding activity 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). Second, in addition to the Sp factors, the CHRNA3/A5/B4 promoter regions can directly interact with and be trans-activated by the more spatially restricted regulatory factors Sox10 and SCIP/Tst-1/Oct-6 (Fig. 2) (Fyodorov and Deneris, 1996; Liu *et al.*, 1999; Yang *et al.*, 1994). Third, the mRNA levels of the CHRNA3/A5/B4 genes are coordinately up-regulated during neural development (Corriveau and Berg, 1993; Levey *et al.*, 1995; Levey and Jacob, 1996) and coordinately down-regulated following denervation (Zhou *et al.*, 1998). Perhaps the most compelling evidence for a coordinated

regulatory scheme comes from the Deneris lab, which showed that two transcriptional regulatory elements, $\beta 43'$ and conserved noncoding region 4 (CNR4), play key roles in directing expression of the clustered nAChR genes in a tissue-specific manner with $\beta 43'$ being important for expression in the adrenal gland and CNR4 being critical for expression in the pineal gland and superior cervical ganglion (Fig. 2)(Xu *et al.*, 2006). CNR4 is likely to play an important role in directing nAChR gene expression in the brain as well (Xu *et al.*, 2006). In addition to these shared regulatory features, the CHRNA3/A5/B4 genes are subject to gene-specific regulation.

3.2.1. CHRNA3—*In vitro* experiments have shown that the paired-like homeodomain transcription factor, PHOX2A, regulates transcription from the $\alpha 3$ promoter (Benfante *et al.*, 2007). PHOX2A does not appear to bind directly to DNA, however, as the DNA-binding domain does not need to be completely intact for PHOX2A to regulate transcription from the $\alpha 3$ promoter (Benfante *et al.*, 2007). Co-immunoprecipitation experiments demonstrate a physical interaction between Sp1 and PHOX2A, suggesting that PHOX2A is tethered to the $\alpha 3$ promoter through its interaction with Sp1, similar to the interactions of Sp1 with homeodomain transcription factors observed in other systems (Shimakura *et al.*, 2006).

As briefly described above, the POU domain factor SCIP/Tst-1/Oct-6 has been shown to positively regulate transcription from the $\alpha 3$ promoter in a cell-type-specific manner (Yang *et al.*, 1994). Similar to PHOX2A, the POU domain factor SCIP/Tst-1/Oct-6 does not require DNA binding for trans-activation of the $\alpha 3$ promoter (Yang *et al.*, 1994). Deletional analysis of the SCIP/Tst-1/Oct-6 transcription factor demonstrated that only the POU domain is needed for trans-activation. Interestingly, this trans-activation does not depend on the presence of an Sp1 motif in the promoter region and is likely occurring through protein-protein interactions with the basal transcription machinery (Fyodorov and Deneris, 1996). The transcription factor Brn-3a also trans-activates the $\alpha 3$ promoter, while the other members of the Brn-3 family, Brn-3b and 3c, modestly repress $\alpha 3$ promoter activity (Milton *et al.*, 1996). The positive regulation by Brn-3a is thought to be a result of protein-protein interaction as the $\alpha 3$ promoter lacks an obvious octamer or octamer-related binding site for Brn-3 factors (Milton *et al.*, 1996).

A transcriptional enhancer has been discovered upstream of the $\alpha 3$ promoter in a region that overlaps with a 3'-untranslated exon of the $\beta 4$ gene (McDonough and Deneris, 1997). This transcriptional enhancer consists of two identical 37-base pair repeats separated by a 6-base pair spacer. The $\beta 4$ 3' enhancer acts as a cell-type-specific enhancer and is capable of enhancing transcription from the $\alpha 3$ promoter in neuronal-like cells, as well as in neurons in SCG cultures (McDonough *et al.*, 2000). The enhancer contains several E-twenty six (ETS) factor-binding sites, the mutation of which dramatically decreases, but does not completely abolish, $\alpha 3$ promoter activity. The ETS-domain binding factor, Pet-1, has been shown to activate reporter gene transcription in a manner that is both cell type- and $\beta 4$ 3' enhancer-dependent (Fyodorov *et al.*, 1998). Taken together, these experiments suggest that Pet-1 interacts directly with the $\alpha 3$ promoter to activate transcription, though it likely requires additional cell-type-specific cofactors. *In vivo* experiments using transgenic mice showed that a larger DNA fragment between the $\alpha 3$ and $\beta 4$ genes, containing both the $\beta 4$ 3' enhancer and the $\alpha 3$ promoter, is capable of directing expression of a reporter gene to several areas of endogenous $\alpha 3$ expression in the brain (Wada *et al.*, 1989; Yang *et al.*, 1997). Surprisingly however, this DNA fragment did not direct reporter gene expression anywhere in the peripheral nervous system, in which the $\alpha 3$ gene is highly expressed (see above), suggesting that elements in this fragment may be acting as repressors or that other sequences are necessary for peripheral expression.

The presence of an intronic repressor element in the fifth intron of $\alpha 3$ has in fact been reported (Fuentes Medel and Gardner, 2007). The sequence of this $\alpha 3$ intron 5 repressor ($\alpha 3I5$) is highly

conserved and is capable of bidirectional repressor activity *in vitro*. Notably, cell-type-specific repression of promoter activity was observed to be more potent in non-neuronal cell lines than in neuronal cell lines (Fuentes Medel and Gardner, 2007). These data suggest that this segment of DNA and the factors with which it interacts function to restrict expression of $\alpha 3$ to neuronal cell types. The protein-DNA interactions that mediate this effect have yet to be elucidated.

While regulation of the $\alpha 3$ subunit gene in neuronal cells has been extensively studied, the mechanisms regulating $\alpha 3$ expression in non-neuronal cells remain largely obscure. Recently, however, our group was the first to report that a transcription factor, the achaete-scute complex homolog-1 (ASCL1), regulates the expression of $\alpha 3$ and $\beta 4$ and modestly of $\alpha 5$ in lung cancer cells (Improgo *et al.*, 2010).

3.2.2. CHRNB5—The $\alpha 5$ promoter region has been described in several genomic contexts including those in rodents and humans. Transcription of $\alpha 5$ occurs in the opposite direction as $\alpha 3$ and $\beta 4$ (Fig. 1), suggesting that in addition to transcription factors that regulate the entire cluster, distinct mechanisms may govern $\alpha 5$ expression. However, apart from the regulatory factors described above that control expression of all three clustered genes, little is known about these mechanisms. SCIP/Tst-1/Oct-6 does not appear to regulate $\alpha 5$ though it regulates $\alpha 3$ and $\beta 4$. Similarly, in lung cells, ASCL1 appears to regulate $\alpha 3$ and $\beta 4$ but not $\alpha 5$ (Improgo *et al.*, 2010). No other transcription factors regulating $\alpha 5$ expression have been reported, underscoring the need for more research effort in this area.

3.2.3. CHRNB4—In addition to the Sp factors, Sox10 and SCIP/Tst-1/Oct-6, the $\beta 4$ promoter is positively regulated by c-Jun (Melnikova and Gardner, 2001). Trans-activation by all of these factors is abolished when the Sp-binding site on the $\beta 4$ promoter (referred to as a CA box) is mutated. Conversely, synergistic activation of the $\beta 4$ promoter is observed when Sp1 is supplied in concert with Sox10, Sp3 or c-Jun (Melnikova and Gardner, 2001; Melnikova *et al.*, 2000a). Co-immunoprecipitation experiments demonstrated that all of these factors physically interact (Melnikova *et al.*, 2000b) and ChIP experiments confirmed that these interactions occur in the context of native chromatin (Scofield *et al.*, 2008). These findings suggest the existence of a positively-acting multi-subunit transcriptional regulatory complex that assembles on the $\beta 4$ promoter. This result is consistent with the hypothesis that Sp1 is critical for transcription from the $\beta 4$ promoter and likely nucleates the regulatory complex that drives expression of the $\beta 4$ gene.

Two additional transcription factors have been shown to interact with the $\beta 4$ promoter, Pura and heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Du *et al.*, 1998; Du *et al.*, 1997). These proteins interact with another motif, the CT box, located directly upstream of the CA box. hnRNP K is capable of repressing Sp factor-mediated trans-activation of the $\beta 4$ promoter (Du *et al.*, 1998) and also physically interacts with Sox10 (Melnikova *et al.*, 2000b). Similar to hnRNP K, Pura physically interacts with Sox10 (Melnikova *et al.*, 2000b). Moreover, Pura and hnRNP K themselves physically interact (Melnikova *et al.*, 2000b). These proteins may participate in the multi-subunit complex described above to modulate expression of the $\beta 4$ gene in the appropriate cellular context. *In vitro* binding experiments demonstrated that each factor binds preferentially to the opposing single strand elements of the CT box, suggesting that some local DNA helix unwinding may occur (Krecic and Swanson, 1999). Interestingly, Pura and hnRNP K have been shown to function together to negatively impact transcription of genes in other systems and the same may be occurring at the $\beta 4$ promoter (Da Silva *et al.*, 2002). *In vivo* experiments have also shown that a 2.3-kb fragment of the $\beta 4$ promoter, containing the CA and CT boxes, is capable of directing reporter gene expression to brain regions that endogenously express $\beta 4$, further supporting the importance of these elements in regulating $\beta 4$ gene expression (Bruschweiler-Li *et al.*, 2010).

4. Role in Pathological States

4.1. Nicotine Addiction

Nicotine addiction is responsible for approximately 5 million deaths per year worldwide and is the most preventable cause of death in the USA (CDC, 2008). Nicotine addiction involves a series of events starting with the initial use of cigarettes, the transition from experimental smoking to regular smoking, and finally dependence on nicotine (Bierut, 2009b). Nicotine dependence is characterized by heavier smoking, early morning smoking, tolerance and withdrawal. Withdrawal symptoms account for the high incidence of relapse in people attempting to quit smoking (Kenny and Markou, 2001).

At the molecular level, nicotine addiction is initiated by the binding of nicotine to nAChRs. This interaction results in increased dopamine (DA) release in the DAergic mesolimbic and mesocortical circuits (Dani and De Biasi, 2001; Dani and Heinemann, 1996), a phenomenon widely associated with reward or reinforcement and drugs of abuse. DAergic projection neurons in the mesocorticolimbic pathway originate in the ventral tegmentum area (VTA) and project to the nucleus accumbens in the ventral striatum and the prefrontal cortex (Laviolette and van der Kooy, 2004). Several nAChR subtypes are robustly expressed in DAergic neurons both at the level of the soma, as well as at presynaptic terminals. Although, of the clustered nicotinic receptor subunit genes, $\alpha 5$ has been clearly identified as a component of nAChRs at DAergic terminals (Grady *et al.*, 2007). Much of what is known regarding nAChR subtypes that are involved in nicotine addiction stems from studies on genetically engineered KO or knock-in mice (Champtiaux and Changeux, 2004). Mice that lack expression of $\beta 2^*$ nAChRs fail to self-administer nicotine and exhibit impaired tolerance to nicotine (Maskos *et al.*, 2005; McCallum *et al.*, 2006; Picciotto *et al.*, 1998). In addition, the predominant partner of the $\beta 2$ nAChR subunit, $\alpha 4$ has also been implicated in nicotine dependence (Marubio *et al.*, 2003; Tapper *et al.*, 2007; Tapper *et al.*, 2004). Selective activation of $\alpha 4^*$ nAChRs by low doses of nicotine in mice expressing $\alpha 4^*$ nAChRs hypersensitive to agonist is sufficient for nicotine reward/reinforcement, sensitization, and tolerance (Tapper *et al.*, 2004). More recently, it was found that mice that do not express $\alpha 6^*$ nAChRs fail to self-administer nicotine (Pons *et al.*, 2008). Expression of the $\alpha 6$ subunit has also been observed in the striatum and about half of $\alpha 6^*$ nAChRs co-assemble with $\alpha 4$ to form receptors with the highest affinity for ACh in the striatum (Champtiaux *et al.*, 2002; Salminen *et al.*, 2007). Because of their high expression in the reward pathway and high affinity for agonist, $\alpha 4\beta 2^*$ nAChRs have been studied extensively in the context of nicotine addiction.

In recent years, linkage analyses, candidate-gene analyses, and large-scale genome-wide association studies (GWAS) have been used to screen hundreds of thousands of SNPs across thousands of individuals in search of genetic variants associated with nicotine dependence (Baker *et al.*, 2009; Berrettini *et al.*, 2008; Bierut, 2009a; Bierut *et al.*, 2008; Caporaso *et al.*, 2009; Chen *et al.*, 2009a; Freathy *et al.*, 2009; Greenbaum and Lerer, 2009; Grucza *et al.*, 2008; Le Marchand *et al.*, 2008; Saccone *et al.*, 2009a; Saccone *et al.*, 2009b; Saccone *et al.*, 2007; Schlaepfer *et al.*, 2008; Schwartz *et al.*, 2009; Spitz *et al.*, 2008; Stevens *et al.*, 2008; Thorgeirsson *et al.*, 2008; Vink *et al.*, 2009; Wang *et al.*, 2009a; Weiss *et al.*, 2008). This has led to the identification of multiple SNPs in chromosome 15q24–25, a region that contains the CHRNA5/A3/B4 gene cluster as well as the iron-responsive element binding protein, a putative protein of unknown function, LOC123688, and the $\alpha 4$ proteasome subunit protein. Of particular interest is the non-synonymous SNP, rs16969968, found in exon5 of the $\alpha 5$ gene. This polymorphism changes an aspartic acid residue into asparagine at position 398 (D398N) in the second intracellular loop of $\alpha 5$. Individuals with one copy of the risk variant have a 1.3-fold increased risk for nicotine dependence while individuals with two copies have almost a 2-fold increase in risk. This SNP appears to influence different aspects of nicotine dependence, as it has been associated with increased risk for heavy smoking as well as increased sensitivity

to the pleasurable effects of nicotine (Sherva *et al.*, 2008; Stevens *et al.*, 2008). In addition, the presence of this SNP may also influence the biophysical properties of $\alpha 5^*$ nAChRs. When expressed in HEK293T cells, $\alpha 4\beta 2\alpha 5D398$ nAChRs exhibited a greater maximal response to nicotine compared to $\alpha 4\beta 2N398$ nAChRs as measured by calcium imaging (Bierut *et al.*, 2008). The association of this particular SNP has been identified in several studies though the majority of them have involved populations of European descent. In populations of African and Asian descent, the risk allele of rs16969968 is rare but is still associated with nicotine dependence (Saccone *et al.*, 2009b; Wu *et al.*, 2009).

How the clustered nAChRs function in the development of addiction is still unknown though recent studies in nAChR KO mice have revealed a potential role for these subunits in nicotine withdrawal. Withdrawal symptoms can be divided into two classes: somatic or physical symptoms, which likely involve both the PNS and CNS; and affective symptoms, which are centrally-mediated and are associated with hypoactivity of DAergic neurons in the reward circuit (Fung *et al.*, 1996). As in humans, rodents chronically exposed to nicotine exhibit both somatic and affective withdrawal behaviors upon cessation. Somatic withdrawal symptoms in rodents are characterized by increased licking, grooming, shaking and scratching (Damaj *et al.*, 2003; Kenny and Markou, 2001; Malin *et al.*, 1994). Rodent affective symptoms include increased anxiety, hypolocomotion, and conditioned place aversion to nicotinic receptor antagonists (Damaj *et al.*, 2003; Jackson *et al.*, 2008a; Suzuki *et al.*, 1996). The initiation and expression of withdrawal is dependent on neuronal nAChRs based on the fact that 1) symptoms can be alleviated by nicotine treatment and 2) symptoms can be precipitated by administration of nicotinic receptor antagonists during chronic nicotine exposure. Mecamylamine, a noncompetitive nicotinic antagonist, precipitates somatic withdrawal symptoms as well as affective symptoms including hypolocomotion, place aversion and anxiety, when injected i.p. in nicotine-dependent mice or rats (Damaj *et al.*, 2003; Malin *et al.*, 1994; Suzuki *et al.*, 1999; Suzuki *et al.*, 1996). The more selective high affinity nAChR competitive antagonist, dihydro- β -erythroidine (DH β E) (Harvey and Luetje, 1996; Harvey *et al.*, 1996), can also precipitate both somatic and affective symptoms suggesting a role for high affinity nAChRs in withdrawal (Malin *et al.*, 1998). Methyllycaconitine (MLA), a relatively selective $\alpha 7$ antagonist (Alkondon *et al.*, 1992; Palma *et al.*, 1996; Ward *et al.*, 1990)(but see (Klink *et al.*, 2001)), precipitates mild somatic, but few affective withdrawal symptoms (Markou and Paterson, 2001). Finally, somatic withdrawal symptoms can be precipitated by hexamethonium, a nicotinic antagonist that does not cross the blood brain barrier suggesting that peripheral nAChRs also play a role in these symptoms (Hildebrand *et al.*, 1997) although similar symptoms can be elicited by injection of antagonist directly into the brain (Salas *et al.*, 2009). Conversely, affective symptoms are not blocked by i.p. injection of hexamethonium indicating that they are mediated by central nAChRs (Hildebrand *et al.*, 1997; Watkins *et al.*, 2000).

Chronic nicotine-treated $\alpha 5$ and $\beta 4$ KO mice display significantly milder mecamylamine-precipitated somatic withdrawal symptoms compared to WT mice (Salas *et al.*, 2004b; Salas *et al.*, 2009). In the CNS, $\alpha 5$ and $\beta 4^*$ nAChRs are robustly expressed in the habenulo-interpeduncular pathway and direct infusion of mecamylamine into either the medial habenula or the interpeduncular nucleus can precipitate withdrawal in mice dependent on nicotine suggesting that the nAChRs in this pathway are critical for the expression of nicotine withdrawal (Salas *et al.*, 2009). However, chronic nicotine treated $\alpha 5$ KO mice still become anxious during withdrawal suggesting that $\alpha 5^*$ nAChRs may not be involved in affective symptoms (Jackson *et al.*, 2008b).

To date, the role of either $\alpha 5^*$ or $\beta 4^*$ nAChRs in nicotine reward and reinforcement is unclear. Nicotine conditions a place preference in $\alpha 5$ KO mice indicating that expression of $\alpha 5^*$ nAChRs may not be necessary for the rewarding properties of the drug (Jackson *et al.*, 2010).

However, these animals not only exhibit a preference for nicotine at similar doses as WT mice, but also at high doses that normally fail to condition a place preference. Additional analysis of the reinforcing properties of nicotine in KO mice that do not express $\alpha 5$ or $\beta 4$ nAChRs and/or in knock-in mice that express the $\alpha 5$ polymorphism identified in the GWAS should yield valuable insight into the molecular mechanisms underlying nicotine addiction.

4.2. Lung Cancer

Lung cancer is the leading cause of cancer-related deaths for both men and women worldwide (ACS, 2009). In terms of incidence, lung cancer is second only to prostate cancer in men and breast cancer in women. Lung cancer can be divided into two main histological types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The latter can be further divided into adenocarcinoma, squamous cell, bronchioalveolar and large cell lung carcinoma.

Tobacco intake is the main risk factor associated with lung cancer. The scientific link between tobacco and lung cancer has been firmly established since the 1950s (Proctor, 2001). This linkage is not surprising given 1) the addictive effects of nicotine that promote continued use of tobacco despite health consequences and 2) the presence of at least 55 carcinogens in tobacco, including nicotine metabolites such as 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) and N-nitrosornicotine (NNN) (Hecht, 1999; Shields, 2002). NNK and NNN form DNA adducts causing mutations that initiate cancer (Hecht and Hoffmann, 1988). Interestingly, these nitrosamines also serve as ligands for nAChRs (Schuller and Orloff, 1998) and along with nicotine and ACh, have been shown to trigger a variety of cancer-related processes as described below.

In 1989, Schuller's group first showed that nicotine and its metabolites stimulates the growth of lung cancer cells (Schuller, 1989). Shortly thereafter, Minna's group reported that nicotine causes apoptotic inhibition (Maneckjee and Minna, 1990). These two groups and many others thereafter have reported the expression of nAChRs in both normal and malignant lung cells, supporting the notion that nicotine stimulates cancer-related processes in a receptor-mediated fashion (Maneckjee and Minna, 1990; Maus *et al.*, 1998; Sartelet *et al.*, 2008; Schuller, 1989; Song *et al.*, 2003; Wang *et al.*, 2001). Moreover, studies have shown that specific nAChR subunits are over-expressed in lung cancer tissue (Lam *et al.*, 2007). In particular, the CHRNA5/A3/B4 gene cluster is over-expressed in SCLC and this over-expression appears to be regulated by ASCL1 (Improgo *et al.*, 2010). ASCL1 is a basic helix-loop-helix transcription factor important in the initiation and development of SCLC (Ball *et al.*, 1993; Jiang *et al.*, 2009; Linnoila *et al.*, 2000; Osada *et al.*, 2005). Regulation of the CHRNA5/A3/B5 locus by ASCL1 suggests a mechanism by which over-expression of ASCL1 in SCLC leads to a corresponding increase in expression of the clustered nAChR subunits, thereby potentiating the proliferative and pro-survival effects of nicotine and other nAChR ligands (Improgo *et al.*, 2010).

Different groups have shown that various nAChR ligands activate distinct cancer-signaling pathways via the $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$ and $\alpha 7$ nAChR subtypes (Schuller, 2009). ACh stimulates cell proliferation by acting as an autocrine growth factor in SCLC, a phenomenon that can be blocked by the non-specific nAChR antagonist, mecamylamine (Song *et al.*, 2003). Nicotine also up-regulates the expression of growth factors and their cognate receptors (Conti-Fine *et al.*, 2000). NNK causes cell proliferation by activating the serine/threonine kinase RAF1 and mitogen-activated protein kinase, leading to the activation of the oncogenic transcription factor c-myc, a process that can be blocked by the $\alpha 7$ -selective antagonist, α -bungarotoxin (Jull *et al.*, 2001). In addition, nicotine and NNK play a role in apoptotic inhibition. Nicotine activates the anti-apoptotic protein BCL-2 and inactivates the proapoptotic proteins Bad and Bax (Jin *et al.*, 2004; Mai *et al.*, 2003; Xin and Deng, 2005). Nicotine and NNK also activate the Akt/

protein kinase B pathway, inhibiting apoptosis and causing tumorigenesis (West *et al.*, 2003). Activation of Akt by nicotine appears to depend on nAChRs containing $\alpha 3$ or $\alpha 4$ whereas NNK activation depends on $\alpha 7$ nAChRs. Finally, nicotine acts as a pro-angiogenic agent, promoting endothelial cell migration, proliferation, tube formation and survival, an effect antagonized by α -bungarotoxin (Cooke and Ghebremariam, 2008; Heeschen *et al.*, 2001). Along with its metabolite, cotinine, nicotine also up-regulates the expression of the vascular endothelial growth factor in endothelial cells (Conklin *et al.*, 2002).

The aforementioned candidate-gene analyses and GWAS have also implicated the CHRNA5/A3/B4 gene cluster in lung cancer. The same SNP associated with nicotine dependence was found to increase the risk for lung cancer (Amos *et al.*, 2008; Hung *et al.*, 2008; Thorgeirsson *et al.*, 2008). In particular, Hung and colleagues analyzed approximately 317,000 SNPs in thousands of patients and controls and found that the non-synonymous SNP, rs16969968, is robustly associated with lung cancer. This association was not affected by smoking status and was observed even in never-smoker groups, suggesting that the association is not due to the influence of nicotine dependence. Furthermore, no increased risk for other smoking-related cancers such as head and neck cancers was observed, indicating that the association with lung cancer is direct (Hung *et al.*, 2008). Two other SNPs, in exon 5 and the 3'-untranslated region of the $\alpha 3$ gene, were also found in these studies to be associated with lung cancer (Amos *et al.*, 2008; Hung *et al.*, 2008; Thorgeirsson *et al.*, 2008).

4.3 Other Diseases

Aside from nicotine addiction and lung cancer, the CHRNA5/A3/B4 locus has also been associated with alcoholism. SNPs in this locus are associated with alcohol dependence and the level of response to alcohol (Chen *et al.*, 2009b; Wang *et al.*, 2009b). Furthermore, the variants appear to influence age at initiation of both tobacco and alcohol use (Schlaepfer *et al.*, 2008). Since alcohol and nicotine are usually co-abused, these associations may provide a genetic mechanism for this co-morbidity (Greenbaum and Lerer, 2009). The risk allele of the non-synonymous SNP, rs16969968, also appears to be protective for cocaine dependence, suggesting a more general involvement of the CHRNA5/A3/B4 gene cluster in the actions of drugs of abuse (Gruzca *et al.*, 2008).

Other smoking-related diseases are influenced by variants in the CHRNA5/A3/B4 locus. Two SNPs in this region are associated with chronic obstructive pulmonary disease, a serious lung disease characterized by chronic inflammation and progressive destruction of lung tissues (Pillai *et al.*, 2009). Interestingly, nicotine has been shown to promote the development of SCLC-like tumors in a rodent model of chronic obstructive pulmonary disease (Schuller *et al.*, 1995). Another study showed that a variant influencing the risk for nicotine dependence and lung cancer also increases the risk for peripheral arterial disease, a condition characterized by obstruction of arteries outside the heart and brain (Thorgeirsson *et al.*, 2008). The association of variants in the CHRNA5/A3/B4 gene cluster on other smoking-related diseases may be an effect of nicotine dependence, suggesting a gene-environment interaction, or may represent a direct effect, indicating pleiotropy of this locus (Bierut, 2009a; Thorgeirsson *et al.*, 2008).

5. Conclusions

Tobacco use continues to be a major global health threat, underscoring the need to understand the mechanisms leading to nicotine dependence and smoking-related diseases such as lung cancer. Nicotine activation of nAChRs in the CNS initiates nicotine dependence. However, it is clear that nAChRs are also expressed outside the nervous system and are likely involved in numerous smoking-related pathologies. Although most studies to date have implicated the high affinity $\alpha 4\beta 2^*$ nAChRs in nicotine dependence, less is known regarding the role of $\alpha 3\alpha 5\beta 4^*$ nAChRs in this disease (Picciotto *et al.*, 1998; Tapper *et al.*, 2004). However, the recent flurry

of GWAS implicating the CHRNA5/A3/B4 gene cluster in nicotine dependence lends greater credence to the involvement of these hitherto understudied nAChR subunits in nicotine addiction (Berrettini *et al.*, 2008; Bierut *et al.*, 2008; Portugal and Gould, 2008; Saccone *et al.*, 2009a; Saccone *et al.*, 2007; Schlaepfer *et al.*, 2008). That results of both hypothesis-free GWAS and hypothesis-driven candidate-gene studies converged on this gene cluster is particularly compelling. These studies underscore the importance of elucidating the mechanism by which $\alpha 3\alpha 5\beta 4^*$ nAChRs may modulate nicotine-mediated behaviors. In particular, future studies should examine nicotine self-administration in the existing mouse nAChR KO models as well as in knock-in mice expressing the polymorphisms identified in the GWAS.

Even more provoking are similar studies implicating this gene cluster in lung cancer (Amos *et al.*, 2008; Hung *et al.*, 2008; Thorgeirsson *et al.*, 2008). It is still somewhat controversial, however, as to whether the association with lung cancer is direct or merely a by-product of the strength of the addiction or smoking behavior (Chanock and Hunter, 2008). As discussed in this Review, several lines of evidence support the hypothesis of a direct role of nAChRs in lung cancer. In particular, a number of pharmacology-based studies have demonstrated that nAChR activation can initiate a variety of signal transduction cascades associated with cancer (Schuller, 2009). However, it is important to acknowledge that the currently available pharmacological reagents used in these studies, while selective, are not specific, thus care must be taken when interpreting the results. This is a particularly important point with respect to the CHRNA5/A3/B4 cluster, for which the availability of selective ligands is extremely limited. Thus, design of highly specific nAChR ligands is of prime importance. Alternatively, studies using RNA interference and knockout/knock-in mouse models should help elucidate the function of specific nAChR subtypes in lung cancer.

Determining the role of the CHRNA5/A3/B4 cluster in nicotine dependence and lung cancer is important for the rational design of therapeutic interventions. In particular, the use of nAChR ligands as smoking cessation therapies have to take into consideration the possible effects of these drugs on lung cancer.

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Fig. 1. nAChR Structure

(A) Schematic representation of an individual nAChR subunit. The amino and carboxy terminals of the protein are labeled N and C, respectively, with the transmembrane segments labeled M1 – M4. (B) Examples of homomeric and heteromeric nAChR subtypes. Individual receptor subunits are represented as colored circles. Diamonds located between adjacent receptor subunits represent ligand-binding sites while unfilled circles in the center of each pentamer represent the pore region. (C) Schematic representation of the human CHRNA5/A3/B4 gene cluster. Each gene is drawn to scale with blue boxes representing exons and red boxes representing untranslated regions. Horizontal black lines represent introns while horizontal gray lines represent intragenic regions. The boundaries for each gene are labeled with corresponding Genbank annotations. Colored arrows indicate the direction of transcription. The recently discovered D398N polymorphism (rs16969968) associated with nicotine dependence and lung cancer is indicated by the arrow below the fifth exon of the CHRNA5 gene

Fig. 2.

Transcriptional regulation of the CHRNA5/A3/B4 gene cluster. (A) Protein-DNA interactions regulate expression of the clustered nAChR subunit genes. Coding regions of the subunits are represented as light green boxes with arrows indicating the direction of transcription. Two transcriptional regulatory elements, the intronic repressor in the fifth intron of $\alpha 5$ (A3I5) and the 3' enhancer in the $\beta 4$ gene, are shown (green and purple boxes, respectively). Horizontal black lines depict the non-coding regions of DNA. Transcription factors that regulate expression of these genes are depicted as colored circles and are touching each other if they directly interact. Circles labeled with a "?" indicate transcription factors whose identities have yet to be identified. In the case of Sp1, multiple binding sites have been identified in each of the nAChR genes, however, for clarity, the sites are represented as a single green circle at each promoter region. (B) Positive and negative regulation of the clustered nAChR subunit genes. Coding regions of the clustered subunits are represented as light green boxes with arrows that indicate the direction of transcription. Four transcriptional regulatory elements are depicted in this figure: A3I5 (green box), the $\beta 4$ 3' enhancer (purple), the SacI – HindIII fragment of the $\beta 4$ promoter region (orange box), and the distal CNR4 regulatory region (yellow box). The boundaries of the latter two regions are labeled relative to the transcriptional start site of the $\beta 4$ gene. Red arrows denote positive regulatory effects whereas blue arrows indicate negative transcriptional regulation.

Table 1

Expression of the CHRNA5/A3/B4 Genes

Location	CHRNA5	CHRNA3	CHRNA4	Reference
CNS				
Brainstem	+	+	nd	(Hellstrom-Lindahl <i>et al.</i> , 1999; Morely, 1997; Wevers <i>et al.</i> , 1994)
Cerebellum	++	+	+	(Flora <i>et al.</i> , 2000a; Hellstrom-Lindahl <i>et al.</i> , 1999; Winzer-Serhan and Leslie, 1997)
Cortex	+	+	+	(Wada <i>et al.</i> , 1990; Hellstrom-Lindahl <i>et al.</i> , 1999; Dineley-Miller and Patrick, 1992)
Habenula	+	nd	+	(Grady <i>et al.</i> , 2009; Winzer-Serhan and Leslie, 1997)
Hippocampus	+++	+	+	(Hellstrom-Lindahl <i>et al.</i> , 1998; Wada <i>et al.</i> , 1990; Winzer-Serhan and Leslie, 1997)
Interpeduncular nucleus	+++	+++	+++	(Grady <i>et al.</i> , 2009; Winzer-Serhan and Leslie, 1997; Dineley-Miller and Patrick, 1992)
Medial habenula	–	+++	+++	(Boulter <i>et al.</i> , 1990; Winzer-Serhan and Leslie, 1997)
Olfactory bulb	nd	+++	+++	(Dineley-Miller and Patrick, 1992)
Pineal gland	nd	+++	+++	(Zoli <i>et al.</i> , 1995; Dineley-Miller and Patrick, 1992)
Substantia nigra	++	++	++	(Azam <i>et al.</i> , 2002)
Spinal cord	+	+	+	(Flora <i>et al.</i> , 2000a; Hellstrom-Lindahl <i>et al.</i> , 1998; Zoli <i>et al.</i> , 1995)
Thalamus	+	+	+	(Flora <i>et al.</i> , 2000a; Hellstrom-Lindahl <i>et al.</i> , 1998)
Ventral tegmental area	++	++	++	(Azam <i>et al.</i> , 2002)
PNS				
Adrenal medulla	nd	+	+	(Rust <i>et al.</i> , 1994; Di Angelantonio <i>et al.</i> , 2003)
Dorsal root ganglia	+	+	++	(Flora <i>et al.</i> , 2000a; Zoli <i>et al.</i> , 1995)
Facial motoneurons	nd	+	nd	(Senba <i>et al.</i> , 1990)
Otic ganglia	nd	++	++	(Rust <i>et al.</i> , 1994; Zoli <i>et al.</i> , 1995))
Retina	+	+	+	(Moretti <i>et al.</i> , 2004)
Sphenopalatine ganglia	nd	++	+	(Rust <i>et al.</i> , 1994; Zoli <i>et al.</i> , 1995))
Superior cervical root ganglia	+	++	++	(Flora <i>et al.</i> , 2000a; Rust <i>et al.</i> , 1994; Zoli <i>et al.</i> , 1995)
Trigeminal ganglia	+	+++	++	(Liu <i>et al.</i> , 1998; Flores <i>et al.</i> , 1996)
Non-Neuronal				
Bronchial epithelium	+	+	+	(Maus <i>et al.</i> , 1998; Wang <i>et al.</i> , 2001)
Gastrointestinal tract	++	+	+	(Flora <i>et al.</i> , 2000a)
Lung	+	+	+	(Flora <i>et al.</i> , 2000a; Improgo <i>et al.</i> , 2010)
T lymphocytes	nd	+	+	(Battaglioli <i>et al.</i> , 1998)
Oral epithelium	+	+	+	(Arredondo <i>et al.</i> , 2001 and 2005)
Oral keratinocytes	nd	+	+	(Arredondo <i>et al.</i> , 2001 and 2005)
02A progenitor	+	+	++	(Rogers <i>et al.</i> , 2001)
Thymus	+	++	nd	(Flora <i>et al.</i> , 2000a)
Testis	+	nd	nd	(Flora <i>et al.</i> , 2000a)
Vascular endothelial cells	+	+	+	(Macklin <i>et al.</i> , 1998; Wang <i>et al.</i> , 2001)

nd = not determined; – = no expression; + = expression; ++ = intermediate expression; +++ = high levels of expression

Table 2

ACh and Nicotine Potency for Various nAChR Subtypes

Subtype	EC50 ACh (mM)	EC50 Nic (mM)	Species	Reference
$\alpha 3\beta 2$	26 – 209	6.8 – 70	Human	(Wang <i>et al.</i> , 1996; Gerzanich <i>et al.</i> , 1998; Nelson <i>et al.</i> , 2001)
$\alpha 3\alpha 5\beta 2$	0.5 – 121	1.9 – 83	Human	(Wang <i>et al.</i> , 1996; Gerzanich <i>et al.</i> , 1998; Nelson <i>et al.</i> , 2001)
$\alpha 3\beta 4$	79 – 180	56 – 106	Human	(Wang <i>et al.</i> , 1996; Gerzanich <i>et al.</i> , 1998; Nelson <i>et al.</i> , 2001; Groot-Kormelink <i>et al.</i> , 2001)
$\alpha 3\alpha 5\beta 4$	81 – 207	42 – 105	Human	(Wang <i>et al.</i> , 1996; Gerzanich <i>et al.</i> , 1998; Nelson <i>et al.</i> , 2001; Groot-Kormelink <i>et al.</i> , 2001)
$\alpha 4\beta 2\beta 3$	0.28 ± 0.26; 48.7 ± 43.1	1.78 ± 0.7	Human	(Kuryatov <i>et al.</i> , 2008)
$\alpha 4\alpha 5\beta 2$	1.23 ± 0.42	0.36 ± 0.081	Human	(Kuryatov <i>et al.</i> , 2008)
$\alpha 4\beta 2$	0.23 – 1.9	0.12 – 0.3	Human	(Kuryatov <i>et al.</i> , 2008; Kuryatov <i>et al.</i> , 2000)
$\alpha 4\beta 2$	0.8	0.8	Chick	(Ramirez-Latorre <i>et al.</i> , 1996)
$\alpha 4\alpha 5\beta 2$	100	~12	Chick	(Ramirez-Latorre <i>et al.</i> , 1996)

Nic = Nicotine; ACh = Acetylcholine; EC50 values were taken from studies discussed in the text

Table 3

Transcription Factors that Regulate the CHRNA5/A3/B4 Genes

Transcription Factor	CHRNA5	CHRNA3	CHRNA4	Reference
ASCL1	nd	yes	yes	(Improgo <i>et al.</i> , 2010)
Brn-3a	nd	yes	no	(Milton <i>et al.</i> , 1996)
c-Jun	nd	nd	yes	(Melnikova <i>et al.</i> , 2001)
hnRNP K	nd	nd	yes	(Du <i>et al.</i> , 1998)
NF-Y	nd	nd	yes	(Valor <i>et al.</i> , 2002)
Pet-1	nd	yes	nd	(Fyodorov <i>et al.</i> , 1997)
PHOX2A	nd	yes	nd	(Benfante <i>et al.</i> , 2007)
Pura	nd	nd	yes	(Du <i>et al.</i> , 1997)
SCIP	nd	yes	yes	(Fyodorov <i>et al.</i> , 1996; Yang <i>et al.</i> , 1994)
Sox10	yes	yes	yes	(Liu <i>et al.</i> , 1999)
Sp1	yes	yes	yes	(Campos-Caro <i>et al.</i> , 1999; Yang <i>et al.</i> , 1995; Bigger <i>et al.</i> , 1996)
Sp3	yes	yes	yes	(Bigger <i>et al.</i> , 1997)

nd = not determined; no = no regulation; yes = regulation by the indicated transcription factor