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Neuronal nicotinic receptors: from structure to function

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Molecular identification of the genes and resulting protein sequences for a large number of nicotinic acetylcholine receptor (nAChR) subunits has stimulated numerous studies highlighting their role in several human behaviors including neurological disorders and nicotine addiction. This receptor gene family is likely to be involved in release of multiple neurotransmitters in both brain and periphery that mediate sensitivity and tolerance to nicotine. Recent findings also suggest that alterations in these receptors may lead to neurological diseases, some associated with increased incidence of smoking. This review addresses current knowledge of nicotinic receptor structure, regulation of expression, and function, in both normal and psychiatric subjects.

Introduction

Expression and function of the nicotinic acetylcholine receptor (nAChR) gene family are likely to be important factors in nicotine addiction. The development of tolerance and eventual addiction to nicotine, as well as the responses to the neuroadaptations that lead to withdrawal symptoms and relapse, are complex and involve multiple neurotransmitter systems. However, the principal effects of nicotine downstream from the cigarette begin with its action at the nicotinic receptor. Interventions, presently available to smokers, do not result in high quit rates at 1 year post-cessation. The addition of antidepressants has made a minor improvement in treatment, but permanent smoking cessation is still rarely achieved, particularly in the mentally ill. Understanding the structure, expression and function of this large gene family and its relationship to other neurotransmitter systems is essential for the formulation of nicotine addiction hypotheses and subsequent treatments that will result in true smoking cessation in both normal subjects and those with psychiatric disorders.

The presence of nicotinic receptors on muscle cells and neurons that display functional differences in response to acetylcholine has been recognized for many years (Ascher, Large, & Rang, 1979; Popot & Changeux, 1984; Rang & Ritter, 1970). However, the gene family is large and the possible subunit combinations numerous. Furthermore, in recent years it has become clear that neuronal nAChRs are widely expressed throughout the body, where they are likely to have diverse but important roles.

To date, 11 genes coding for neuronal nAChR subunits have been identified from the mammalian genome ($\alpha2$–$\alpha7$, $\alpha9$, $\alpha10$, and $\beta2$–$\beta4$), reviewed in Lukas and Bencherif (1992), McGehee and Role (1995), Elliott *et al.* (1996), Gotti, Fornasari, and Clementi (1997), and also see GenBank Accession Number AF196344. Phylogenetically linked to the broader family of four transmembrane ligand-gated channels, which include the γ-aminobutyric acid ($GABA_A$ and $GABA_C$), the glycine and serotonergic receptors ($5\text{-}HT_3$), nicotinic receptors are thought to be among the first of the ligand-gated ion channels to evolve, long before evolution of vertebrates and their complex nervous systems (Le Novère & Changeux, 1995). The nicotinic receptor gene family is even larger in invertebrates (Ballivet, Alliod, Bertrand, & Bertrand, 1996; Jonas, Phannavong, Schuster, Schroder, & Gundelfinger,

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1994; Schulz *et al.*, 1998; Squire *et al.*, 1995). Since at least 16 genes for nAChR subunits are believed to be expressed in *Caenorhabditis elegans* (Brownlee & Fairweather, 1999), it is possible that additional subunit genes remain to be discovered in vertebrates. In that regard, the sequence for a novel rat neuronal nicotinic receptor cDNA (α10), was recently released by Jim Boulter, UCLA (GenBank Accession Number: AF196344). A human ortholog has also been identified (F. Sgard and P. Fuchs, personal communication).

Neuronal receptors are thought, as their muscle counterparts, to result from the assembly of five subunits around an axis of pseudosymmetry (Bertrand & Changeux, 1992; Miyazawa, Fujiyoshi, Stowell, & Unwin, 1999; Unwin, 1996). Each of the neuronal subunits is likely to span the membrane four times with the second transmembrane domain forming the wall of the ionic pore (Bertrand & Changeux, 1992). While overall structures of the proteins forming the neuronal nicotinic receptors appear quite similar, their genes and exon–intron relationships differ markedly (Lindstrom, 1996). The acetylcholine (ACh) binding sites and membrane spanning regions (MI–IV) are highly conserved between subunit types, but the large cytoplasmic loop between MIII and MIV is unique to each subunit and conserved across species (Elliott *et al.*, 1996; McGehee & Role, 1995). A comparison of exon/intron borders for a subset of the nicotinic receptor subunit gene family is shown in Figure 1A. The exon/intron junction pattern is more complex for the α7 subunit, which has 10 exons, than for the other subunits, which have five or six. Further, two of these splice junctions, unique to the α7 subunit, are found near the border of MI and within the coding region for MII, the latter thought to face the ion channel (Corringer, Le Novère, & Changeux, 2000; Galzi, Revah, Bessis, & Changeux, 1991). The location of the exon/intron splice sites in the α7 gene, relative to the amino acid sequence of MI and MII are shown schematically in the assembled pentamer in Figure 1B.

Heterologous expression studies have revealed that functional neuronal nAChRs can be divided in two groups: (a) the homomeric receptors and (b) the heteromeric receptors (Weiland, Bertrand, & Leonard, 2000). Expression of α7, α8 or α9 subunits alone has been shown to result in functional receptors, while all the other subunits require at least a pair wise expression of an α and β combination (Elgoyhen, Johnson, Boulter, Vetter, & Heinemann, 1994; Gerzanich, Anand, & Lindstrom, 1994; Kuryatov, Olale, Cooper, Choi, & Lindstrom, 2000; Luetje & Patrick, 1991). The newly identified α10 subunit is more homologous to α7 and α9 than to the α subunits that bind nicotine with high affinity (α4, α3). Attempts to express α10 alone yielded no functional receptors but co-expression with its closest homolog α9 caused a marked difference in α9 receptor properties that more closely resembled those of the native receptor in outer hair cells (F. Sgard, personal communication). This suggests that α9 and α10 probably assemble in the same receptor complex.

Although the α7-receptor subunit may be expressed in the brain as an homomer (Drisdel & Green, 2000), whether it always occurs in this conformation *in vivo* is unclear. Functionally distinct, α7 containing receptors exist in chick sympathetic neurons that may contain other subunits (Girod *et al.*, 1999; Yu & Role, 1998b). It was also recently reported that while neurons from chick cardiac ganglia express a receptor containing the α7 subunit they display a pharmacological profile distinct from that of homomeric receptors reconstituted in *Xenopus* oocytes or in cell lines (Cuevas & Berg, 1998). It is possible that co-assembly of the α7 subunit with other nicotinic receptor subunits is region or tissue specific and may depend on the relative ratio or type of subunits expressed (Nelson & Lindstrom, 1999; Yu & Role, 1998a).

It is, perhaps, not surprising that the α7 subunit is principally expressed as an homomer, since it was probably the first subunit in the gene family to evolve, long before development of the brain. Major rearrangements in the neuronal nicotinic receptor genes have occurred during evolution by gene duplication and mutation, resulting in the variable exon content (Le Novère & Changeux, 1995) and different regulatory mechanisms. The earliest nicotinic receptor subunit gene is thought to be the α7 gene, which maps to human chromosome 15q13–q14. A partial duplication of the α7 gene (*dupα7*) has been cloned and maps less than 1Mb proximal to the full-length α7 gene. *Dupα7* is expressed as mRNA with novel exons and is alternatively spliced, as is the mRNA for the full-length α7 gene (Gault *et al.*, 1998). Three other nicotinic receptor subunit genes map distally at chromosome 15q24 (α3, α5 and β4), and form a gene cluster (Boulter *et al.*, 1990; Couturier *et al.*, 1990a). Three subunits of the muscle nicotinic receptor map near each other on chromosome 2 (Lobos, 1993). Table 1 summarizes our current knowledge of the human chromosomal localization of the neuronal nicotinic receptor subunit genes.

Localization of gene expression

Compared to other ligand-gated ion channels, gene expression of the neuronal nicotinic receptors is generally low in the brain. Expression for each subunit varies during development and across brain regions. The most abundant subunits expressed in human brain are the α4, α7, and β2 subunits. The highest levels of β2 expression were found in caudate, putamen, and hippocampus, while α3 expression was highest in thalamus (Gotti *et al.*, 1997; Rubboli *et al.*, 1994). The α4 subunit was most highly expressed in the cortex (Agulhon *et al.*, 1998; Wevers *et al.*, 1994), while α7 expression was highest in human lateral and medial geniculate and the reticular thalamic nucleus (RTN; Agulhon, Abitbol, Bertrand, & Malafosse, 1999; Breese *et al.*, 1997a; Wevers *et al.*, 1995). Expression of α5 is generally low in the human central nervous system (CNS), except for cerebellum and thalamus, where

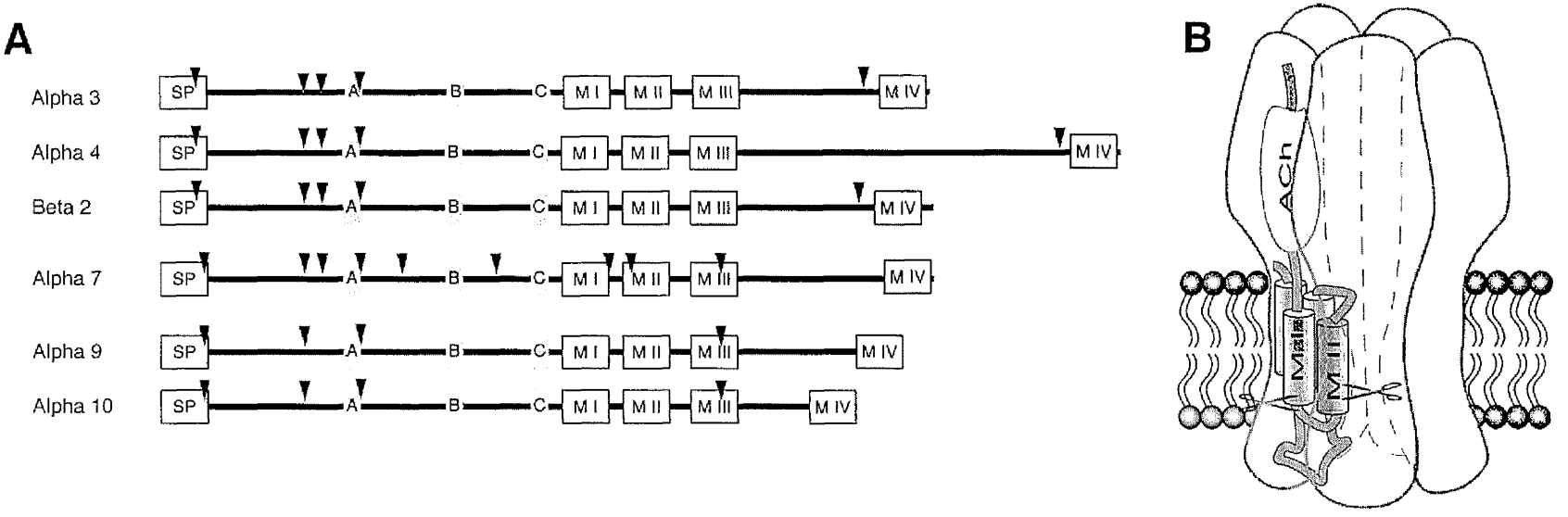

Figure 1. Schematic representation of splice junctions for neuronal nicotinic acetylcholine receptor subunit genes and an assembled α7 homomeric receptor. (A) Exon/intron junctions for a subset of the nicotinic acetylcholine receptor subunit gene family. SP, signal peptide; MI to MIV, membrane spanning regions I to IV; A,B, and C, three domains of the ACh binding site. Arrows indicate the intron positions (Elgoyhen, Johnson, Boulter, Vetter, & Heinemann, 1994; Steinlein, Weiland, Stoodt, & Propping. 1996; Rempel, Heyers, Engels, Sleegers, & Steinlein, 1998; Gault *et al.*, 1998; F. Sgard, personal communication). (B) Representational structure of an assembled α7 homomeric nicotinic receptor. The ACh binding site is indicated and scissors are positioned at the sites of introns 7 and 8 in the coding region of the gene. Intron 7 lies near the coding region for MI, intron 8 within the coding region for MII, and intron 9 in the coding region for MIII.

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Table 1. Chromosomal localization for the human nicotinic acetylcholine receptor gene family. Published map locations are shown

Receptor subunit	Subunit type	Map location	Reference
α1	Muscle	2q24-q32	Beeson *et al.*, 1990; Lobos *et al.*, 1993
β1	Muscle	17p12-p11	Beeson *et al.*, 1990; Lobos *et al.*, 1993
γ	Muscle	2q33-q34	Beeson *et al.*, 1993; Lobos *et al.*, 1993
δ	Muscle	2q33-q34	Beeson *et al.*, 1990; Lobos *et al.*, 1993
ε	Muscle	17q13-p12	Beeson *et al.*, 1993; Lobos *et al.*, 1993
α2	Neuronal	8p21	Wood *et al.*, 1995
α3	Neuronal	15q24	Boulter *et al.*, 1990; Raimondi *et al.*, 1992
α4	Neuronal	20q13.2-q13.3	Steinlein *et al.*, 1994
α5	Neuronal	15q24	Boulter *et al.*, 1990; Raimondi *et al.*, 1992
α7	Neuronal	15q13-q14	Chini *et al.*, 1994; Orr-Utreger *et al.*, 1995
β2	Neuronal	1q21.3	Lueders *et al.*, 1999
β3	Neuronal	8p11.2	Koyama *et al.*, 1994
β4	Neuronal	15q24	Boulter *et al.*, 1990; Raimondi *et al.*, 1992

moderate levels were observed (Flora *et al.*, 2000a). A disparity in regional expression between the human and rat genes has been noted for α7 in the RTN (Breese *et al.*, 1997a), and for α5 in cerebellum and thalamus (Flora *et al.*, 2000a). Although the pattern of subunit expression is highly conserved across species, disparities between rodents and mammals for expression of the α3 and α2 subunits have been reported (Cimino, Marini, Fornasari, Cattabeni, & Clementi, 1992; Han *et al.*, 2000). These results suggest that species divergence in gene expression has occurred that might affect neuronal circuitry. This possibility merits consideration when pathways regulating behaviors are being studied in multiple species.

It has been generally accepted that the neuronal nicotinic receptor gene family was mainly expressed in the brain and ganglionic synapses of the peripheral nervous system, although a broader localization has recently been suggested. Subunits of principal importance in the chick ciliary ganglia and adrenal chromaffin cells are α3, β4, and α7 (Lukas, Norman, & Lucero, 1993; McGehee & Role, 1995; Vernallis, Conroy, & Berg, 1993; Vijayaraghavan, Pugh, Zhang, Rathouz, & Berg, 1992). Expression of the α7 subunit has also been found in developing muscle (Romano, Pugh, McIntosh, & Berg, 1997). Multiple nicotinic receptor subunits, including α3, α5, α7, β2, and β4 are expressed in keratinocytes, bronchial epithelial cells, and arterial endothelium (Grando *et al.*, 1995; Horton, Nguyen, & Grando, 1997; Macklin, Maus, Pereira, Albuquerque, & Conti-Fine, 1998; Maus *et al.*, 1998; Zia, Ndoye, Nguyen, & Grando, 1997). A recent survey of α5 mRNA expression in human peripheral tissues indicates this receptor subunit is also expressed in the gut and in the thymus (Flora *et al.*, 2000a,b). In cultured bronchial epithelial cells, nicotine regulates both the integrity of cell shape and motility (Conti-Fine, Navaneetham, Lei, & Maus, 2000).

Nicotinic receptors are found in both lymphocytes and polymorphonuclear (PMN) cells of human peripheral blood where their function is not yet known (Benhammou *et al.*, 2000; Hiemke *et al.*, 1996; Lebargy *et al.*, 1996). In some peripheral tissues, cells have been found to express both a cholinergic phenotype and to have nAChRs on their surface; function is thought to involve autocrine or paracrine mechanisms (Nguyen *et al.*, 2000; Wessler, Kirkpatrick, & Racke, 1999). Thus, receptors responding to nicotine have a wide expression pattern and may have non-synaptic roles in addition to those in the CNS.

Receptor assembly

Nicotinic receptor subunits are known to undergo post-translational modification by glycosylation and phosphorylation (Chen, Dang, & Patrick, 1998). Glycosylation has been shown to be necessary for binding of some ligands (Luetje, Maddox, & Harvey, 1998) and perhaps for correct folding of the peptide (Blount & Merlie, 1990; Rickert & Imperiali, 1995), but may not be required for receptor assembly (Chen *et al.*, 1998). However, subunits that do not acquire ligand-binding capacity may be rapidly degraded (Blount & Merlie, 1990), affecting efficient receptor assembly (Green & Millar, 1995). Indeed, nicotinic receptor assembly appears to be exquisitely sensitive to correct folding; only 30% of synthesized subunits in the muscle become incorporated into competent surface receptors (Blount & Merlie, 1990).

Although several stable cell lines expressing human neuronal nicotinic receptors have been developed (Buisson, Gopalakrishnan, & Bertrand, 1999), obtaining competent receptor expression of α7 receptors in stably transfected cell lines is considered to be more difficult than for other subunits. Additionally, receptor expression in the transfected cultures is not generally uniform. Studies using chimaeras of the α7 subunit, usually with the 5-HT_3 receptor that is also a ligand-gated ion channel, suggest that the membrane-spanning regions may contribute to the low assembly efficiency for this receptor (Cooper, Harkness, Baker, & Millar,

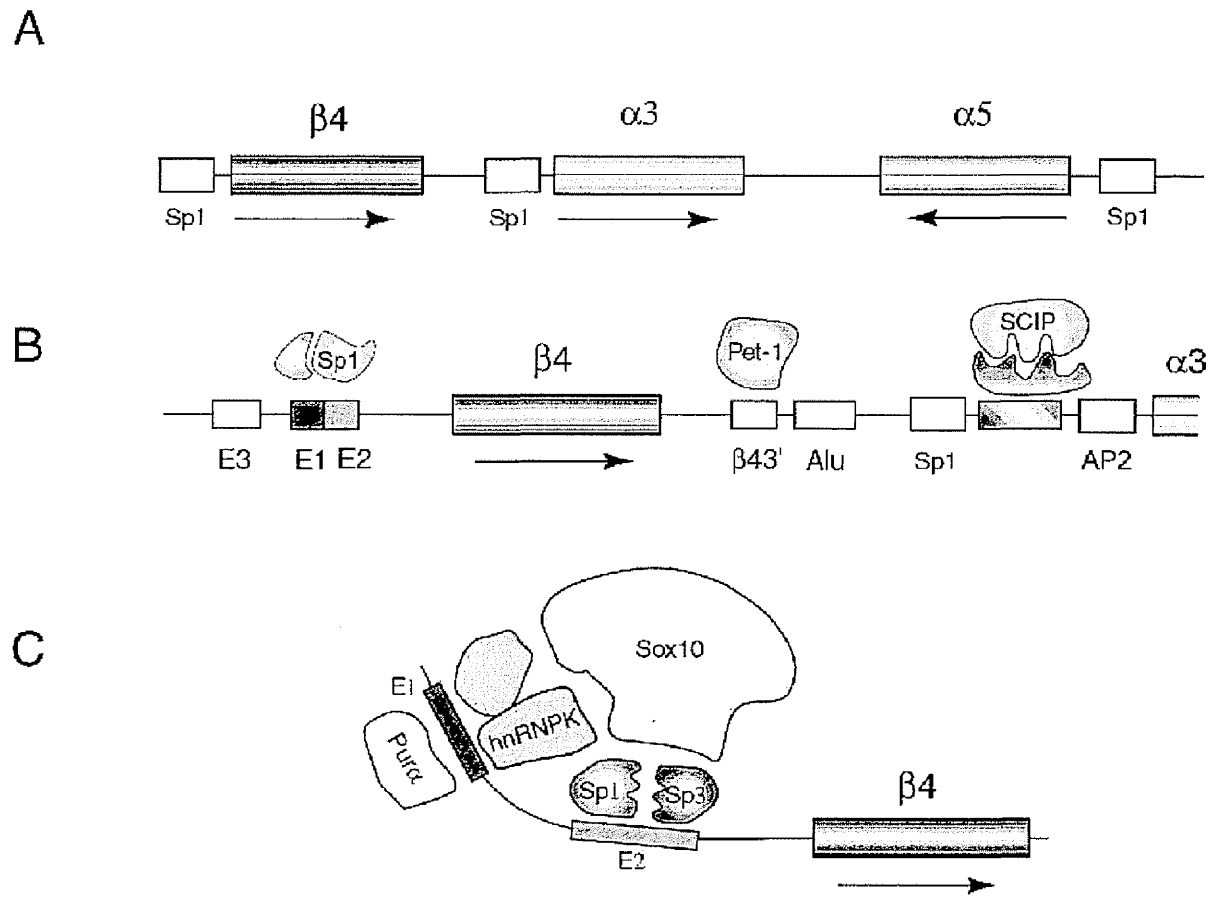

Figure 2. Schematic representation of the β4, α3, α5 gene cluster. (A) Map of the β4, α3, α5 gene cluster. Arrows indicate the transcription polarities. (B) Enlargement of β4 gene map showing the principal known promoters and enhancer elements. (C) Schematic drawing of the β4 transcription regulatory elements.

1999; Corringer *et al.*, 1995; Eisele *et al.*, 1993). Indeed, specific amino acids in the α7 subunit have been found to control receptor distribution between the cell surface and intracellular pools (Dineley & Patrick, 2000). Amino acids near and within membrane-spanning region I (MI) were found to affect surface receptor expression. Since overexpression, or a significant increase in function of the α7 receptor has been shown to result in increased neuronal cell death (Berger, Gage, & Vijayaraghavan, 1998; Orr-Urtreger *et al.*, 2000), the tight regulation of functional surface expression demonstrated for the α7 receptor may be important in development.

Despite the stringency apparently required for competent surface α7-receptor expression and the fact that the stability of α7-receptor expression in cultured cell lines derived from tumors has also been questioned (Kassner & Berg, 1997), a rat α7 cDNA has been successfully expressed in several cell lines including rat pituitary (Quik, Choremis, Komourian, Lukas, & Puchacz, 1996), neuronally derived human cell line SH-SY5Y (Puchacz, Buisson, Bertrand, & Lukas, 1994), and a human α7 clone in embryonic kidney cell line HEK 293 (Gopalakrishnan *et al.*, 1995).

Regulation of gene expression

Levels of gene expression and tissue specificity are thought to be principally determined by regulatory elements in the promoter and 5′-untranslated regions (UTRs) of genes. The diversity of transcription factor binding sites, enhancer and repressor sequences in the nicotinic receptor subunit genes, thus far characterized, suggest exquisite and generally individualized regulation by these promoter elements (Fornasari, Battaglioli, Terzano, & Clementi, 1999). The β4 and α3 genes lie in a gene cluster on human chromosome 15q24, which also includes α5 (Table 1; Figure 2A). Conservation of this cluster suggests there are likely to be shared regulatory mechanisms (Boulter *et al.*, 1990). All three of these genes are GC-rich and have promoter elements that bind the transcription factor Sp1 (Bigger, Casanova, & Gardner, 1996; Fornasari, Battaglioli, Flora, Terzano, & Clementi, 1997), as do many of the nicotinic receptor subunit gene promoters (Fornasari *et al.*, 1999). Elements that coordinately regulate the expression of the α3 and β4 genes have been identified in the region between the two genes. A neuron-selective enhancer, the β43′, functions in both orientations and coordinately activates

both the α3 and β4 genes, which are transcribed in the same direction (Fyodorov, Nelson, & Deneris, 1998; McDonough & Deneris, 1997). The β43′ enhancer also appears to be activated by ETS factors such as Pet-1 (Figure 2B; Deneris *et al.*, 2000), as was found for muscle subunits γ and ε (Schaeffer, Duclert, Huchet-Dymanus, & Changeux, 1998).

Transcription of the α5 gene is known to occur in the opposite direction to that of α3 and β4 and thus α5 may not share promoter or enhancer elements with the other two genes in the cluster. The α5 gene is expressed in both neuronal and non-neuronal tissues and the 5′-UTR of the α5 promoter can act as either an activator or an inhibitor of gene expression in neuronal or non-neuronal cells, respectively (Flora *et al.*, 2000b). Expression of the α5 subunit is observed as early as E11 in the chick. Homeodomain transcription factor Phox 2B, necessary for neurogenesis in the autonomic nervous system (Pattyn, Morin, Cremer, Goridis, & Brunet, 1999), activates α3, but not α5.

A core promoter for the β4 gene of 226 bp has been isolated, containing both a CT-box binding Purα and hnRNPK (Du, Tomkinson, & Gardner, 1997), and a CA-box binding transcription factors Sp1 and Sp3 (Figure 1C; Bigger, Melnikova, & Gardner, 1997; Du, Melnikova, & Gardner, 1998). The binding site for another factor, Sox 10, overlaps the CT and CA boxes (Melnikova, Yang, & Gardner, 2000). In addition to Sp1 sites, the α3 promoter is activated by the Pou domain transcription factor SCIP, apparently through a protein–protein interaction (Deneris *et al.*, 2000).

A core promoter of 143 bp has been isolated for the β3 gene (Matter *et al.*, 1998), containing an E box that binds basic helix–loop–helix (bHLH) proteins. Specifically, the *atonal* homolog cATH5, whose expression is principally confined to developing retina, activates the β3 core promoter more than 10-fold. The time courses of expression during development for both cATH5 and β3 are similar.

The sequences for the bovine (Carrasco-Serrano, Campos-Caro, Viniegra, Ballesta, & Criado, 1998) and human (Gault *et al.*, 1998) α7 gene promoters have been reported. The two core promoter sequences are similar; both are GC-rich and contain several Egr-1 transcription factor consensus sites, as well as a CREB site, suggesting that the promoters may be steroid regulatable. This is consistent with results in the mouse, showing that the α7 gene is regulated by neurosteroids (Bullock *et al.*, 1997; Pauly, Grun, & Collins, 1990). The Egr-1 component of transcription factor binding to the bovine α7 promoter is increased in bovine adrenomedullary cells on treatment with phorbol esters, indicating that the α7 gene may be a specific target for *egr-1* gene expression (Carrasco-Serrano, Viniegra, Ballesta, & Criado, 2000).

Although functional investigation of regulatory elements for most of the nicotinic receptor subunit genes is far from complete, there are similarities and differences between the elements controlling the expression of the different subunits. Differences are expected to play a fundamental role during development and tissue specific activation, likely controlling the gene specific roles of the subunits. For example, since it is known that the α3β4 combination is the most abundant in ganglionic receptors, coordinated regulation for the expression of these two genes may be expected in the autonomic system.

Function

One of the interesting features of the neuronal nAChRs is their localization in both the brain and periphery, and how this relates to function. Although individual nAChR subtypes may have a more restricted localization, one or more subtypes are found in most tissues. Observations made on rat brain synaptosomes have shown that application of nicotinic agonists can provoke the release of dopamine (Grady, Marks, Wonnacott, & Collins, 1992; Grady, Marks, & Collins, 1994; Misu *et al.*, 1993; Wonnacott, 1997). However, strong evidence suggests that nicotinic receptors are expressed both pre- and post-synaptically, and in complex patterns that include both axo-axonic and somato-dendritic synapses (Amador & Dani, 1995; Bordey, Feltz, & Trouslard, 1996; Dolezal, Lee, Schobert, & Hertting, 1996; Guo, Tredway, & Chiappinelli, 1998; Lena & Changeux, 1997; MacDermott, Role, & Siegelbaum, 1999; Marchi & Raiteri, 1996; McGehee & Role, 1996; Ullian & Sargent, 1995). Additionally, the nicotinic receptor subunit gene family appears to be growing with the discovery of the α10 subunit (Boulter *et al.*, GenBank Accession Number AF196344; F. Sgard personal communication), and it is not yet known whether all of the subunits have been cloned. With the completion of the genome sequencing efforts, this question should be resolved in the near future.

Examination of the physiological properties of heterologously expressed nicotinic receptors, composed of the known subunits, reveals large differences between receptor subtypes. At one extreme are the receptors that desensitize slowly, composed of α4β2 subunits (Bertrand, Ballivet, & Rungger, 1990; Buisson, Gopalakrishnan, Arneric, Sullivan, & Bertrand, 1996) while at the other extreme is the fast desensitizing α7 receptor (Couturier *et al.*, 1990b; Gopalakrishnan *et al.*, 1995; Séguéla, Wadiche, Dineley-Miller, Dani, & Patrick, 1993; Vijayaraghavan *et al.*, 1992; Zhang, Vijayaraghavan, & Berg, 1994). Receptors composed of α3β4 or α3β4α5 display a slower time course of desensitization than α7 receptors (Couturier *et al.*, 1990a; Role, 1992).

Such differences have also been observed in brain receptors measured either on cultured cells or in slices (Alkondon & Albuquerque, 1993, 1995; Alkondon *et al.*, 1994; Alkondon, Rocha, Maelicke, & Albuquerque, 1996; Frazier, Buhler, Weiner, & Dunwiddie, 1998a; Frazier *et al.*, 1998b; Lena *et al.*, 1999; Zaninetti,

Tribollet, Bertrand, & Raggenbass, 1999; Zoli, Lena, Picciotto, & Changeux, 1998). Differences in pharmacological properties have been further documented *in vivo* (Decker, Brioni, Bannon, & Arneric, 1995). Speculations concerning the role of the nicotinic receptors, therefore, depend upon the subtype considered, the tissue in which they are expressed, and the subcellular localization.

High sensitivity to nicotine and sustained responses, which are characteristic of the major brain $\alpha4\beta2$ receptor, result in prolonged tonic responses while low sensitivity and highly desensitizing receptors typical of $\alpha7$ are thought to provoke a fast, phasic depolarization (Boulter *et al.*, 1987; Monteggia *et al.*, 1995), and for $\alpha7$ (Couturier *et al.*, 1990b; Peng, Katz, Gerzanich, Anand, & Lindstrom, 1994; Séguéla *et al.*, 1993). Responses of either $\alpha4\beta2$ or $\alpha7$ receptors are accompanied by a significant increase in the intracellular calcium concentration (Bertrand, Galzi, Devillers, Bertrand, & Changeux, 1993; Haghighi & Cooper, 2000; Ragozzino, Barabino, Fucile, & Eusebi, 1998; Rogers & Dani, 1995; Sands, Costa, & Patrick, 1993; Tsuneki, Klink, Lena, Korn, & Changeux, 2000; Vernino, Amador, Luetje, Patrick, & Dani, 1992). Thus, for receptors expressed pre-synaptically it can be expected that they play a role in modulating the response of the synaptic bouton, and either directly or indirectly increasing the intracellular calcium concentration. Elevation of the intracellular calcium concentration increases the probability of neurotransmitter release. Indeed, as demonstrated by several groups, neuronal nAChRs play a major role in controlling release of many different neurotransmitters (Guo *et al.*, 1998; Lena & Changeux, 1997; McGehee & Role, 1995; Pidoplichko, DeBiasi, Williams, & Dani, 1997; Wonnacott, 1997). In contrast, fast synaptic transmission caused by neuronal nicotinic receptors has been demonstrated in only a few cases in the CNS, but is expected to play an important role that awaits further characterization (Alkondon, Pereira, Eisenberg, & Albuquerque, 1999; Frazier *et al.*, 1998a, b; Hefft, Hulo, Bertrand, & Muller, 1999; Roerig, Nelson, & Katz, 1997). Additional modulatory roles of nicotinic receptors can also be anticipated in view of their properties and subcellular localization. For example, it is widely documented that nAChRs are expressed on the cell soma and ACh-evoked currents have been classified (Albuquerque *et al.*, 1995; Lena *et al.*, 1999; Yakel & Jackson, 1988; Zaninetti *et al.*, 1999; Zoli *et al.*, 1998). Stimulation of these receptors is expected to cause effects both through cell depolarization and/or increase of the intracellular calcium concentration. Evidence of calcium modulation has already been provided for NMDA receptors (Fisher & Dani, 2000). The effect of nAChR activation in stratum radiatum interneurons was recently found to induce either inhibition or disinhibition of pyramidal neurons that may be dependent on activation of specific receptor subtypes (Ji & Dani, 2000).

Recent reports also suggest that single cells can express more than one receptor subtype. In approximately 30% of the ventral tegmental (VTA) dopaminergic neurons, both fast and slow desensitizing currents have been found, indicating that these neurons may express both $\alpha7$ and $\alpha4\beta2$ receptors (Pidoplichko *et al.*, 1997). In contrast, only 4% of neurons in the substantia nigra display these two types of responses. The activation and desensitization kinetics in the VTA varied significantly with low concentrations of nicotine, altering the firing of the neurons on both a shorter and a longer time scale (Pidoplichko *et al.*, 1997). In this same brain area it was also shown that activation of nicotinic receptors on presynaptic terminals enhanced glutamatergic input to dopaminergic neurons (Mansvelder & McGehee, 2000). From the sensitivity to methyllycaconitine (MLA) these investigators concluded that both short- and long-term effects of nicotine required activation of an $\alpha7$-containing receptor. Additional evidence for the contribution of $\alpha7$ receptors to the intracellular calcium concentration was also provided in mouse brain slices (Tsuneki *et al.*, 2000).

In rat superior cervical ganglia two types of $\alpha7$ containing, MLA sensitive receptors have been characterized (Cuevas, Roth, & Berg, 2000). One class has fast desensitization properties similar to homomeric $\alpha7$ receptors. However, the second class, also blockable by MLA, desensitized slowly and differed in the reversibility of α-bungarotoxin blockade.

In the striatum, pharmacological evidence for two types of nicotinic receptors on dopaminergic terminals has also been found. Using α-conotoxin-MII, which has been shown to display a high selectivity for $\alpha3\beta2$ and the anatoxin-a/epibatidine hybrid UB-165, $\alpha3\beta2$ and $\alpha4\beta2$ receptors were found to play a role in dopamine release (Kaiser, Soliakov, Harvey, Luetje, & Wonnacott, 1998; Sharples *et al.*, 2000). Dopamine release was partially blocked by $\alpha7$ antagonists and glutamate antagonists, suggesting that the $\alpha7$ effect may be modulated through glutamate release onto dopaminergic terminals (Kaiser & Wonnacott, 2000). However, *in vivo* glutamate release may be dependent on nicotine dose; at low nicotine concentrations, tonic activation of NMDA receptors in the VTA may be necessary for dopamine release (Fu, Matta, Gao, Brower, & Sharp, 2000). The major subunit expressed in the mesolimbic system appears to be the $\alpha6$ receptor (Goldner, Dineley, & Patrick, 1997; Le Novère *et al.*, 1999; Le Novère, Zoli, & Changeux, 1996). Since the physiological and pharmacological characterization of $\alpha6$-containing receptors remains to be determined, evidence regarding the specificity of the α-conotoxin-MII effects on $\alpha6$-containing vs. $\alpha3\beta2$ nAChRs has not yet been firmly established.

Evidence for more than one receptor subtype on GABAergic neurons from cultured rat hippocampus and in hippocampal slices from both human and rat has been reported (Albuquerque, Pereira, Alkondon, Schrattenholz, & Maelicke, 1997; Alkondon, Pereira, & Albuquerque, 1998; Hilmas, Alkondon, Eisenberg, &

Albuquerque, 1999). Acetylcholine induced both a rapidly decaying current and a slowly decaying whole-cell current; the latter was sensitive to dihydro-β-erythroidine (DHβE). The fast current was likely to be α7 mediated, as it was blocked by MLA, and the slow was likely an α4β2 receptor. Approximately 45% of interneurons in hippocampus may exhibit ACh currents, mediating effects on GABA release by both receptor subtypes.

A further complexity in evaluating the effects of neuronal nAChRs is their possible direct interaction with other ligand-gated channels. For example, occlusion of P2X responses by neuronal nAChR activation has been documented by several laboratories (Johnson, Shum, Thornton, & Bornstein, 1999; Searl, Redman, & Silinsky, 1998; Zhou & Galligan, 1998). Co-activation of α3β4 nAChR and P2X receptors results in cross-inhibition of both channel types (Khakh, Zhou, Sydes, Galligan, & Lester, 2000). These authors have demonstrated that inhibition was observed only at high channel density and was found to be state dependent.

Localization of α7 receptors on dendritic spines associated with cytoskeletal elements has been found in chick ciliary ganglia, where apparent function may involve receptor stability and prevention of run-down. The clustering on the spines may have the effect of protecting the cell from rapid Ca^{2+} influx. Intracellular injection of the calcium-chelating agent BAPTA caused a significant reduction of the response. Run-down appears to be under the influence of calmodulin, through a calmodulin kinase II pathway. Blockade of the phosphatase calcineurin increased run-down (Chang & Berg, 1999; Conroy & Berg, 1999; Liu & Berg, 1999). The actin cytoskeleton has been shown to be important for retaining these receptor-rich spines (Shoop, Yamada, & Berg, 2000).

Pharmacology

Although acetylcholine is the principal endogenous ligand for the neuronal nicotinic receptors, other pharmacological agents may activate or inhibit the receptors. It is important to recall that while α4β2 receptors display a sensitivity to ACh and nicotine in the micromolar range (Buisson *et al.*, 1996), α7 receptor half activation is observed only at about 150 μM for ACh and 40 μM for nicotine (Gopalakrishnan *et al.*, 1995). In slices of both human cerebral cortex and rat hippocampus, choline (10 mM) induced an inward current that decayed to baseline prior to the termination of the agonist pulse and was sensitive to 50 nM MLA (Alkondon, Pereira, Cortes, Maelicke, & Albuquerque, 1997; Alkondon *et al.*, 1999). Lower concentrations (1 mM) have been found to potentiate the effects of ACh at α4β4 receptors expressed in oocytes (Zwart & Vijverberg, 2000). Choline concentration in the CSF ranges between 4 and 12 μM (Klein, Gonzales, Koppen & Loffelholz, 1993), suggesting that choline may not affect the response of endogenous nAChR receptors. However, choline concentration at the synapse and in the vicinity of perisynaptic receptors is not known and may reach millimolar concentrations immediately following acetylcholine hydrolysis. Several compounds have been found that appear to modulate nicotinic receptors. Galanthamine is a reversible, competitive cholinesterase inhibitor that allosterically increases activity of nicotinic receptors and has shown some promise in treatment of Alzheimer's disease (Maelicke & Albuquerque, 2000; Raskind, Peskind, Wessel, & Yuan, 2000; Tariot *et al.*, 2000). Nefiracetam, and its parent alkaloids piracetam and aniracetam, have been recently shown to facilitate hippocampal neurotransmission, as well (Oyaizu & Narahashi, 1999; Nishizaki *et al.*, 2000; Nomura & Nishizaki, 2000). The facilitatory action was blocked by antagonists of both high- and low-affinity nicotinic receptors, suggesting that these compounds might be effective at multiple receptor subtypes.

Substance P and other tachykinins also interact with multiple nicotinic receptor subunits (Cuevas & Adams, 2000; Lukas & Eisenhour, 1996; Valenta, Downing, & Role, 1993). Substance P has been shown to inhibit epibatidine stimulated release of catecholamine from chromaffin cells with an IC_{50} of 5 μM (Krause, Michael, Lubke, Livett, & Oehme, 1997). Receptor inhibition was found to be non-competitive and to involve the β subunits (Stafford, Oswald, Figl, Cohen, & Weiland, 1998; Stafford, Oswald, & Weiland, 1994). The α7-receptor inhibition by substance P was similar to that of the high-affinity nicotinic receptors. Although substance P inhibition was always observed in the micromolar range, this interaction may play an important physiological role as suggested by the recent findings in rat cardiac ganglia neurons (Cuevas & Adams, 2000).

Several studies suggest that endogenous neurosteroids may be allosteric inhibitors of nicotinic receptors (Bertrand, Valera, Bertrand, Ballivet, & Rungger, 1991; Ke & Lukas, 1996; Valera, Ballivet, & Bertrand, 1992). Progesterone was found to be an inhibitor of α4β2 receptors, while A-ring metabolites of progesterone appear to also inhibit α3-containing receptors that regulate dopamine release (Bullock *et al.*, 1997). A recent comparison of several other neurosteroids indicated that compounds with the β-orientation of groups at the C-17 position are more potent than the α-oriented diastereomers (Paradiso, Sabey, Evers, Zorumski, Covey, & Steinbach, 2000). The most powerful allosteric modulation was reported for the antihelminth ivermectin on the α7 receptor (Krause *et al.*, 1998). When exposed to low concentrations of this compound, human or chick α7 receptors displayed an increase in their sensitivity to ACh by two orders of magnitude.

Cocaine has been found to selectively inhibit specific nAChR subtypes with a potency in the μM range. The α4 and β4 subunits appear to be targeted with specific residues implicated (Francis, Vazquez, Papke, & Oswald, 2000). A structural analog of morphine, dextromethor-

phan, blocked nicotine-stimulated $Rb^{86}(+)$ efflux in a non-competitive manner in membranes prepared from a stable nAChR-transfected human embryonic kidney cell line (Hernandez *et al.*, 2000). This drug is widely used as a cough suppressant.

Another group of drugs that have been shown to interact with nicotinic receptors are antidepressants. Serotonin reuptake inhibitors such as sertraline and fluoxetine inhibit nicotinic receptor function in a non-competitive manner in TE671/RD and SHSY-5Y cell lines (Fryer & Lukas, 1999a). Bupropion and phencyclidine act in a similar manner (Fryer & Lukas, 1999b). Several of these drugs have shown promise in smoking cessation trials (Benowitz & Peng, 2000).

Many other drugs are expected to interact with the nicotinic receptors. For example, it was documented that both memantine and amantadine are extremely potent open-channel blockers at the human $\alpha4\beta2$ receptors (Buisson & Bertrand, 1998). Modulatory sensitivity of the neuronal nicotinic receptor family by this wide range of pharmacological agents suggests that there is much yet to learn concerning the endogenous regulation of these ion channels, and effects on release of multiple kinds of neurotransmitters in both brain and periphery.

Nicotine addiction

Understanding the processes underlying nicotine addiction requires characterization of the neuroadaptive effects of sustained exposure to nicotine, on both nicotinic receptors and the other neurotransmitter systems affected by nicotinic receptor activation and desensitization. The paradoxical up-regulation of high-affinity nicotinic receptors by nicotine has been recognized for some time in both rodent brain (Flores, Davila-Garcia, Ulrich, & Kellar, 1997; Marks, Stitzel, & Collins, 1986; Pauly, Marks, Robinson, van de Kamp, & Collins, 1996; Sanderson, Drasdo, McCrea, & Wonnacott, 1993) and more recently in human brain (Benwell, Balfour, & Anderson, 1988; Breese *et al.*, 1997b; Perry, Davila-Garcia, Stockmeier, & Kellar, 1999) and human blood leukocytes (Benhammou *et al.*, 2000). This up-regulation of receptor number is dose dependent (Benhammou *et al.*, 2000; Breese *et al.*, 1997b; Marks, Stitzel, & Collins, 1986). Low-affinity nicotinic receptors, measured by $[^{125}I]\alpha$-bungarotoxin binding, are increased in rodents at higher nicotine concentration than required for high-affinity receptor up-regulation (Marks, Stitzel, & Collins, 1986). In human brain, the low-affinity $\alpha7$ receptor is expressed at low levels, making accurate ligand binding determinations difficult. A study from Court *et al.* (1998) showed no increase in $[^{125}I]\alpha$-bungarotoxin binding in smokers compared to non-smokers. However, Breese *et al.* (2000) found increased $[^{3}H]$MLA binding in smokers, although the difference between smokers and non-smokers was not significant. These results suggest that in many human smokers, nicotine concentrations in the brain may not reach levels that induce receptor up-regulation. The actual role that receptor up-regulation in smokers plays in the process of nicotine addiction and cessation or relapse is not known. Receptor increases following chronic nicotine administration in animals do not appear to be due to increases in subunit transcription (Pauly *et al.*, 1996), but were rather attributed to increased stability of the receptor itself (Peng, Gerzanich, Anand, Whiting, & Lindstrom, 1994b). It has been suggested that the nicotine-bound receptor may assume an inactivated conformation that is more stable, but is not functional following nicotine binding (Dani & Heinemann, 1996).

Most receptor subunit combinations are generally up-regulated by chronic nicotine treatment (Bhat, Turner, Selvaag, Marks, & Collins, 1991; Peng, Gerzanich, Anand, Wang, & Lindstrom, 1997; Sanderson *et al.*, 1993). However, receptor increases have been found to be both brain region-specific and may be restricted to defined subunit combinations (Rogers, Mandelzys, Deneris, Cooper, & Heinemann, 1992). It has recently been found that the β subunit may affect desensitization and receptor up-regulation. Receptors formed from $\alpha3\beta2$ subunits in transfected cells are up-regulated by chronic nicotine; $\alpha3\beta4$ receptors desensitize to a lesser extent and are not increased by chronic nicotine in this *in vitro* model. It is clear from these results that the subunit combinations expressed can regulate tissue and cellular responses to nicotine.

Activation of nicotinic receptors evokes the release of multiple types of neurotransmitters, including dopamine, norepinephrine, GABA, glutamate, acetylcholine, and neuropeptides (Guo *et al.*, 1998; Wonnacott, 1997). It is likely that neuroadaptations of these neurotransmitter systems, including their receptors, contribute to the development of tolerance to nicotine and the maintenance of addiction (Watkins, Koob, & Markou, 2000).

Dopamine pathways in the pedunculopontine nucleus, VTA, and nucleus accumbens have been implicated in the positive reinforcement provided by nicotine (Balfour, Benwell, Birrell, Kelly, & Al-Aloul, 1998; Nisell, Nomikos, & Svensson, 1994). Nicotine stimulated release of dopamine in the nucleus accumbens is blockable by NMDA receptor antagonists, suggesting that glutamate neurotransmission is also involved in reward pathways. Differential effects of nicotine have been noted in the nucleus accumbens core and shell (Nisell, Marcus, Nomikos, & Svensson, 1997). These disparate responses may be due to reciprocal changes that occur in the medioventral shell and the laterodorsal core (Bassareo & Di Chiara, 1999). In rats chronically treated with nicotine, a sensitization to nicotine responses occurs in the core, but not in the shell. Conversely, dopamine release in accumbens, in response to acute nicotine treatment, is likely to be from the shell. The development of sensitization in the core is thought to strengthen nicotine associations leading to addiction (Balfour *et al.*, 1998; Balfour, Wright, Benwell, & Birrell, 2000; Di Chiara, 2000).

Drugs that directly affect receptor function may be useful in smoking cessation treatment. The antidepressants bupropion and nortriptyline have already been used in this regard (Balfour & Ridley, 2000; Benowitz & Peng, 2000; Prochazka *et al.*, 1998). The nicotinic receptor antagonist, mecamylamine, has also been proposed as a therapy for smoking cessation (Rose, Behm, & Westman, 1998). A recent study suggests that mecamylamine can inhibit the cardioacceleratory and epinephrine-releasing effects of nicotine, likely reducing receptor binding and distribution of coadministered nicotine (Zevin, Jacob, & Benowitz, 2000).

Cytochrome P450–2A6 (CYP2A6) is the principal cytochrome P450-metabolizing enzyme for nicotine (Messina, Tyndale, & Sellers, 1997). Subjects with null alleles for *cyp2A6* increase the bioavailability of nicotine and, thus, smoke less (Pianezza, Sellers, & Tyndale, 1998). Inhibitors of CYP2A6, such as methoxsalen, reduced nicotine metabolism and resulted in decreased smoking in a recent study, suggesting that nicotine metabolism may be an important target for development of smoking cessation interventions (Messina *et al.*, 1997; Sellers, Kaplan, & Tyndale, 2000a; Sellers, Zeman, Kaplan, & Tyndale, 2000b).

New tools for nicotinic receptor research

A need for better tools to dissect the different receptor subtypes constitutes one of the important challenges for both basic science and applied pharmaceutical research. An expanding group of toxins that have differential effects at subtypes of nicotinic receptors are the snail toxins from *Conus geographicus* better known as α-conotoxins (α-CTX). These short peptides of less than 20 amino acids display a high selectivity for the different nAChR subtypes (McIntosh, Santos, & Olivera, 1999). For example, α-CTX MI displays a high affinity for the muscle type receptor but a low affinity for the homomeric α7 receptor. In contrast, the α-CTX IMI from the worm *Conus imperialis* is selective for the α7 receptor (Johnson, Martinez, Elgoyhen, Heinemann, & McIntosh, 1995). Analysis of different toxins and their spectrum of activity on receptor subtypes suggests that α-CTX MII displays a preferential activity for α3β2 nAChRs (Cartier *et al.*, 1996; Whiteaker, McIntosh, Luo, Collins, & Marks, 2000a). The primary mechanism accounting for the large differences observed between toxin affinities seems to reside in their specific off rates.

Extensive studies of neuromuscular transmission have revealed that numerous natural toxins are interacting with the nAChRs expressed by the muscle cell but less with ganglionic receptors. While sensitivity to the snake toxin α-bungarotoxin was used for some time as a characterizing tool for neuronal nAChR, it was later demonstrated that this snake toxin can also exhibit differences in affinity to receptor subtypes. Investigation of the short vs. long snake toxins (Erabutoxin/Cobratoxin) revealed that, while these two toxins bind almost equally to the muscle receptor, only the long toxin displays a high affinity at the α7 nAChR (Servent *et al.*, 1997).

Further investigation of natural products interacting with the nAChRs has shown that these toxins can be divided into two main classes by function, as inhibitors or activators of the receptors. Plant toxins include nicotine, cytisine, and MLA, whereas animal toxins include epibatidine and anabaseine. Originally purified from the equatorial frog skin (*Epipedobates tricolor*; Badio & Daly, 1994), epibatidine was found to be one of the most potent agonists of the heteromeric neuronal nAChRs. Epibatidine binds to several subsets of nicotine receptors with different affinities for cytisine (Marks, Smith, & Collins, 1998). Alpha-CTX MII binds with high affinity to one of these subsets with a low cytisine affinity, resolving epibatidine binding into three populations. These are the high-affinity binding sites representing α4β2 receptors, and the two low-affinity cytisine sites identified by alpha CTX-MII, likely containing α3 subunits (Whiteaker *et al.*, 2000a).

Understanding the differences between an agonist and a competitive antagonist is also an important issue in the field of ligand-gated channels. The analysis *in vitro* of chimaeric receptors where presence or absence of binding sites can be engineered continues to be helpful in this regard (Corringer *et al.*, 1995). Analysis, based on the allosteric model originally formulated by Monod, Wyman, and Changeux (1965), revealed that agonists are compounds that stabilize the active state whereas competitive antagonists are defined by the functional state that they stabilize. For example, while DHβE and MLA are both competitive inhibitors of the α7 receptor, DHβE was shown to stabilize a desensitized state whereas MLA stabilizes the resting state (Bertrand *et al.*, 1997). Newly synthesized compounds such as epibatidine derivatives can be similarly analyzed by functional comparisons (Bertrand *et al.*, 1999; Spang *et al.*, 2000). Although presented as clear-cut differences between agonists and antagonists, it should be recalled that some compounds, including epibatidine, may display complex pharmacological patterns that vary in efficacy at different receptor subtypes. A typical and further complexity of the pharmacological profile is illustrated by the partial agonist behavior of some compounds. A recent illustration of the partial agonist effect of epibatidine was provided in studies of the rat α4β2 nAChRs (Buisson, Vallejo, Green, & Bertrand, 2000). While of extreme importance for drug selectivity, an extensive discussion of pharmacological profile would require an entire review and is not further developed herein.

A series of genetic manipulations in mice has allowed the production of knockouts of specific nAchR genes (Orr-Urtreger *et al.*, 1997; Picciotto *et al.*, 1995; Ross *et al.*, 2000; Xu *et al.*, 1999), as well as the first knock-in of a mutated α7 gene (Orr-Urtreger *et al.*, 2000). These provide valuable tools for study of receptor function in live mammalian systems (Cordero-Erausquin, Marubio, Klink, & Changeux, 2000). The first published nicotinic

receptor knockout was the disruption of the gene coding for the β2 subunit (Picciotto *et al.*, 1995). Designed to suppress the most abundant high-affinity nicotinic receptors, these studies revealed surprisingly little change in behavior of the mouse. The most important alteration, observed thus far in the β2 knockout, is modification of passive avoidance. Further, the β2 knockout mouse does not self-administer nicotine to the same extent as the wild-type mouse, suggesting that β2-containing receptors (presumably α4β2), are likely to be important in the reinforcing properties of nicotine (Picciotto *et al.*, 1998).

Rubidium efflux studies of β2 null-mutants show that the β2 subunit appears to be a component of all receptors that modulate dopamine and GABA release. Acetylcholine release, however, which was detectable in the inferior colliculus and interpeduncular nucleus (IPN) of the β2 null-mutant may be modulated by an α3β4-type receptor (Whiteaker *et al.*, 2000b).

The development of a null-mutant for the α4 gene showed that loss of this subunit results in a reduction of the nicotine antinociceptive effects (Marubio *et al.*, 1999), confirming initial observations about the antinociceptive effects of nicotinic agonists in the CNS. An independently generated knockout of the α4 nicotinic receptor subunit has recently been reported, and has been examined in several behavioral paradigms (Ross *et al.*, 2000). Increases in exploratory behavior, as well as heightened anxiety, were found in these mice. It was suggested that the α4 subunit might be required for activation of inhibitory neural circuits. As anxiety and motor restlessness are associated with nicotine withdrawal (Hughes, Higgins, & Bickel, 1994; Jorenby *et al.*, 1996; McKenna & Cox, 1992; Shiffman *et al.*, 2000), this mouse may be useful in drug development for smoking cessation.

Knockouts of α3 and α7 genes have also been generated (Orr-Urtreger *et al.*, 1997; Xu *et al.*, 1999). Suppression of α3 gene expression considerably reduced survival; only one-third of the homozygous littermates survived but displayed megacystis as well as mydriasis. Attributed to the alteration of the ganglionic nicotinic receptor activity, these impairments caused marked modification of the bladder size, apparition of bladder stones, and incontinence (De Biasi, Nigro, & Xu, 2000; Xu *et al.*, 1999). Mice lacking the α7-subunit gene show only subtle phenotypic changes. The baroreflex appears to be affected (Franceschini *et al.*, 2000) and the mice exhibit increased hippocampal excitability (Orr-Urtreger *et al.*, 1997).

While conditional knockouts may provide a better insight into the role of nicotinic receptors in the CNS, alternative information can be obtained using knock-ins with mutated receptors. A mutation in the MII region of the α7-receptor subunit, equivalent to the chick L247T mutation originally described by Revah *et al.* (Bertrand *et al.*, 1992; Revah *et al.*, 1991), has been recently introduced into a tissue culture cell line, resulting in cell death (apparently due to a gain of function that causes an excessive entry of calcium; Briggs *et al.*, 1999). Treatment of a cultured fetal hippocampal cell line with nicotine has been shown to induce apoptosis, mediated by the α7 nicotinic receptor (Berger *et al.*, 1998). The mechanism was again thought to be calcium mediated, due to a lack of calcium buffering in the fetal cells, suggesting that nicotine is apoptotic at very early embryonic stages and may be neuroprotective later in development. The α7 MII mutation has recently been introduced into a transgenic animal where it results in lethality in the first 24 h of life (Orr-Urtreger *et al.*, 2000). Electrophysiological investigations of cultured hippocampal neurons from these mice confirmed the gain of function in the homozygous animal with an increase of the ACh sensitivity, decrease of desensitization, and conversion of competitive antagonists into agonists. These results suggest that regulated expression of the α7 receptor during development may be important for programmed cell death, and perhaps neuronal migration. In that regard, prenatal administration of nicotine has been shown to induce aberrant lung development. In a primate model, prenatal nicotine resulted in lung hypoplasia and reduced surface complexity of the developing alveoli. Increases in expression of α7-containing receptors were also noted (Sekhon *et al.*, 1999).

To examine in more detail the contribution of α9 in hearing function, Vetter and collaborators have developed a mouse knockout of this gene (Vetter *et al.*, 1999). Morphological evidence has confirmed a localization of the α9 receptor in the outer cochlear hair cells (Elgoyhen *et al.*, 1994). In the α9 knockout, mature innervation of these hair cells was lost and resulted in the failure of suppression of cochlear responses, following fiber activation.

The importance of nAChRs in disease

Nicotinic receptors may play a role in several human diseases, as well as in cognition. If not causal, the involvement of nicotinic receptor function, in many cases, is likely to be important. The first disease in which nicotinic receptors were carefully studied was myasthenia gravis (Beeson *et al.*, 1998; Croxen *et al.*, 1997; Vincent *et al.*, 1998). In this autoimmune disease, antibodies against the muscle receptors are developed causing either a progressive reduction of the receptor number or blockade of neurotransmission. In both cases muscle weakness with excessive muscle fatigue is observed together with impairment of muscle control. A few rare cases of genetically transmissible myasthenia gravis have been reported, and to date at least 14 mutations have been found for the muscle receptors in this disease (Engel *et al.*, 1996; Ohno *et al.*, 1995, 1996, 1997; Ohno, Anlar, Ozdirim, Brengman & Engel, 1998). Electrophysiological investigations of receptors reconstituted with these altered subunits have further revealed that mutations are accompanied by a reduction of receptor function that can explain the diminution of synaptic transmission.

Genetic linkage of a disease to the chromosomal map site of a nicotinic receptor has been used to implicate nicotinic receptor roles in two diseases, autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) and schizophrenia. A gene locus was assigned for ADNFLE to chromosome 20q13.2 the locus of the α4 nicotinic receptor subunit. Subsequently a point mutation leading to a change in an amino acid (Ser248Phe) was identified in the second transmembrane region of the α4 subunit in the linkage family (Steinlein *et al.*, 1995). Another α4-Ser248Phe-linked ADNFLE family has been found, but was genetically distinct from the first pedigree, based on other inherited markers (Steinlein *et al.*, 2000). A second type of mutant with an insertion of an amino acid into the extracellular end of the second transmembrane region of CHRNA4 that reduces the calcium permeability has been identified (Steinlein *et al.*, 1997). Both types of mutants lead to hypoactivity of α4-containing nAChRs (Bertrand, Weiland, Berkovic, Steinlein, & Bertrand, 1998). While supporting the linkage between a mutation in the α4 subunit and ADNFLE, these data also illustrate the importance of the second transmembrane segment. Another epilepsy, juvenile myoclonic epilepsy (JME) has been linked to the gene locus of the α7 nicotinic receptor on chromosome 15q14 (Elmslie *et al.*, 1997). JME is a distinctive and common form of familial idiopathic generalized epilepsy.

The locus of the α7-subunit gene at 15q13-q14 (Table 1) has also been genetically linked to schizophrenia in several independent studies. A polymorphic marker, less than 100 kb from the α7 nicotinic receptor gene, was linked [logarithm of the odds ratio (lod) score of 5.3, $\Theta = 0.00$] to an auditory gating deficit in schizophrenia (Freedman *et al.*, 1997). Linkage to schizophrenia was also found in a second cohort by the same group (Leonard *et al.*, 1998a), and has been replicated in several laboratories (Craddock *et al.*, 1999; Kaufmann *et al.*, 1998; Riley *et al.*, 2000; Stassen *et al.*, 2000). The α7 gene is partially duplicated and the duplication maps within the linkage region on chromosome 15, making it a candidate gene also (Gault *et al.*, 1998). Schizophrenic subjects have been reported to have reduced expression of the α7-receptor subunit in hippocampus (Freedman, Hall, Adler, & Leonard, 1995), in the RTN (Court *et al.*, 1999) and in frontal cortex (Guan, Zhang, Blennow, & Nordberg, 1999). It is of interest that a related disorder, manic depression, has recently been genetically linked to this same locus (Edenberg *et al.*, 1997).

Schizophrenics have also been shown to have reduced levels of high-affinity nicotinic receptors in several brain regions, as measured by either [^{3}H]nicotine (Breese *et al.*, 2000) or by [^{3}H]cytisine (Durany *et al.*, 2000; Freedman *et al.*, 1995). These decreases are not likely to be a result of typical neuroleptic medication (Lee, Breese, & Leonard, 2000). The low levels of both high- and low-affinity nicotinic receptors in schizophrenia may be related to the very high incidence of smoking seen in this disease (Dalack, Healy, & Meador-Woodruff, 1998; Glassman, Covey, Dalack, & Stetner, 1992; Hughes, Hatsukami, Mitchell, & Dahlgren, 1986; Leonard *et al.*, 1998b). Nicotine normalizes a sensory gating deficit found in schizophrenics that is thought to be related to their inability to filter out extraneous or intrusive information in their environment (Adler, Hoffer, Griffith, Waldo, & Freedman, 1992; Adler, Hoffer, Wiser, & Freedman, 1993; Adler, Smas, Fiandaca, Frim, & Majzoub, 1990; Freedman, Waldo, Bickford-Wimer, & Nagamoto, 1991). It has been suggested that smoking is, thus, an attempt at self-medication in which patients are stimulating a reduced population of nicotinic receptors (Adler *et al.*, 1998; Leonard *et al.*, 1998b). The atypical neuroleptic clozapine, which also normalizes this gating deficit (Nagamoto, Adler, Hea, Griffith, McRae, & Freedman, 1996), appears to decrease smoking in schizophrenic patients (McEvoy, Freudenreich, McGee, Vanderzwaag, Levin, & Rose, 1995). The incidence of smoking in depression is also higher than in subjects with no mental illness and smoking cessation attempts are less successful (Glassman *et al.* 1990, 1992). In depression, the effects of nicotine are thought to involve modulation of stress-induced responses that return when the subject is not smoking (Balfour & Ridley, 2000).

Compounds that may be therapeutically specific for the α7 nAChR have been isolated as derivatives of the snail toxin anabaseine, such as GTS-21 (Li, Papke, He, Millard, & Meyer, 1999; Meyer, King, & Meyers, 1998; Meyer *et al.*, 1997; van Haaren, Anderson, Haworth, & Kem, 1999). These drugs appear to protect cells from a range of necrotic and apoptotic signals by increasing calcium influx over a narrow concentration range, and mediating the activation of protein kinase C. GTS-21 has been found to normalize an auditory evoked-potential deficit in mice that have decreased levels of α7 expression (Stevens, Kem, Mahnir, & Freedman, 1998), similar to the auditory deficit found in most schizophrenics that is normalized by nicotine (Adler *et al.*, 1992, 1993; Freedman *et al.*, 1991; Leonard *et al.*, 1998b). These results suggest that GTS-21 might also normalize gating deficits in human subjects.

The nicotinic receptor gene family may be important in both Alzheimer's and Parkinson's diseases. Expression, of both high- and low-affinity nicotinic receptor subunits, declines with aging (Court *et al.*, 1997; Marutle, Warpman, Bogdanovic, & Nordberg, 1998). In Alzheimer's disease, however, there are further selective reductions that appear to be region specific. The α4 subunit was found to be reduced in temporal cortex, but α7 was selectively lost from the RTN where α7 is particularly abundant (Court *et al.*, 1999). High-affinity binding to α7 receptors of the β-amyloid peptide (Aβ 1–42) suggests a role for nicotinic receptors in Alzheimer's disease (Wang *et al.*, 2000a; Wang, Lee, Davis, & Shank, 2000b). Nicotinic-receptor expression is also affected in Parkinson's disease (Perry *et al.*, 1995). In MPTP-treated squirrel monkeys (*Saimiri sciureus*), a Parkinson's disease model, α6 and β3 subunits were selectively decreased, suggesting that receptor ligands directed to these subunits might be useful in treatment of this disorder (Quik & Jeyarasasingam, 2000).

Tourette's syndrome responds to transdermal nicotine therapy and the treatment appears to be long lasting. Motor and vocal tics present in the disease are decreased by nicotine given as either gum or patch (Shytle, Silver, Philipp, McConville, & Sanberg, 1996), and the effects can last up to 4 weeks following the drug application (Dursun & Reveley, 1997). Recently, a 2-year trial using the high-affinity antagonist mecamylamine has shown significant effect on tics and other behavioral disturbances in Tourette's, suggesting that peripheral nicotinic receptor antagonists may be useful in this disease (Silver, Shytle, & Sanberg, 2000).

In a common smoking-associated lung cancer, small cell lung carcinoma (SCLC), the release of autocrine growth factors serotonin and mammalian bombesin/gastrin-releasing peptide are regulated by a neuronal nicotinic receptor containing α7 subunits (Codignola *et al.*, 1994; Schuller, 1991; Schuller, Nylen, Park, & Becker, 1990). Both nicotine and NNK, a nicotine-derived nitrosamine, increased Ca^{2+} influx and activated a Raf-1 MAP kinase pathway in fetal pulmonary neuroendocrine and SCLC cells in culture, leading to increased DNA synthesis. Thus, *in utero* exposure of the fetal lung to nicotine or NNK could be an etiological factor in the development of smoking-associated pediatric lung disorders.

Cognition is likely to also be a target for therapeutic approaches involving the nicotinic receptor gene family. Although improvement of cognitive performance by nicotine in both human and rat is widely accepted, its mode of action still remains obscure. Responses to visual stimuli are improved by nicotine (Levin, Bettegowda, Weaver, & Christopher, 1998; Levin & Simon, 1998). Nicotine patches at 7 mg/day in human subjects significantly reduced the number of errors and increased consistency of cognitive responses. Nicotine has also been shown to produce a small but significant improvement in the overall cognitive performance of Alzheimer's patients (White & Levin, 1999; Potter *et al.*, 1999). The competitive antagonist DHβE at 6 mg/kg blocks the effects induced by 0.05–0.15 mg/kg of nicotine on attention in an animal model, a nicotine concentration compatible with that found in human plasma (Mirza & Stolerman, 1998; Shoaib & Stolerman, 1999; Stolerman, Mirza, Hahn, & Shoaib, 2000). In contrast, MLA, a specific competitive antagonist of α7 receptors, was inefficient, indicating that nicotine effects were probably mediated through high-affinity receptors.

Two compounds from Targacept (Winston-Salem, North Carolina), RJR-2483 and RJR-1784 have effects on memory. Broad screening of these substances revealed that RJR-2483 resembles nicotine, is a full agonist at the α4β2 nAChR, improves both short- and long-term memory and has long-lasting effects (Abdulla *et al.*, 1996; Papke, Bencherif, & Lippiello, 1996; Summers, Lippiello, & Giacobini, 1996; Bencherif *et al.*, 1996). Additionally, at 0.1 μM/kg, RJR-1734 causes a major improvement in the performance of rats placed in a radial arm maze compared to RJR-2483. Effects lasting as long as 18 h following a single oral administration could be observed, supporting the hypothesis that compounds acting on the nicotinic receptor may become of clinical relevance. Compounds from SIBIA may also be effective in cognition (Cosford *et al.*, 1996; Sacaan *et al.*, 1997). SIB-1508 displayed a higher specificity for α4β2 receptors than did SIB-1553. The latter compound is more selective for the α4β4 nAChRs. Broad cognitive improvements have been reported for the SIB-1553, whereas SIB-1508 could bring more benefit in the control of locomotor activity.

Since it is known that the ligand-binding pocket in the nAChRs is made at the interface between the α subunit and its adjacent subunit, it is reasonable to think that ligands specific for a given receptor composition can be developed (Corringer *et al.*, 1995). Compounds with an inter-nitrogen distance of 4.79 Å have been examined (Decker & Meyer, 1999). One group of derivatives was constructed from the 3-pyridil-ethers which conserve some of the features of the natural agonist epibatidine. These compounds display a subnanomolar affinity for α3- or α4-containing receptors, comparable to that of the natural toxin epibatidine (Abreo *et al.*, 1996; Badio & Daly, 1994). Design of drugs specifically targeted to one receptor subtype is well illustrated by the identification of ABT594 (Bannon *et al.*, 1998). Designed to mimic the antinociceptive effects of epibatidine while displaying lower toxicity, ABT594 was shown to significantly relieve pain in animal models. Contribution of the α4β2 receptor in the pain pathway was later confirmed in the α4 knockout mouse model (Marubio *et al.*, 1999).

Future prospects

Although progress in understanding the role that neuronal nAChRs play in neuronal function, nicotine addiction, and disease has been remarkable, a monumental task yet remains for this field. An exhaustive search must be made for other receptor subunits, both in the brain and in the periphery, before we can begin to assign specific functions to subunit combinations *in vivo*. Continued development of new ligands with defined specificities for subunit combinations will provide new tools for pharmacologists and for informed drug development for smoking cessation. The study of gene regulation for this family of receptor subunits is just beginning, as our knowledge of promoter structure and transcription factor interactions grows. Lastly, the finding of nicotinic receptor involvement in multiple illnesses suggests that nicotinic-receptor mutations might play a role through interaction with other gene products and environmental factors to define a disease.

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