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Nicotinic Acetylcholine Receptors in Health and Disease

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Abstract

Nicotinic acetylcholine receptors (AChRs) are a family of acetylcholine-gated cation channels that form the predominant excitatory neurotransmitter receptors on muscles and nerves in the peripheral nervous system. AChRs are also expressed on neurons in lower amounts throughout the central nervous system. AChRs are even being reported on unexpected cell types such as keratinocytes. Structures of these AChRs are being determined with increasing precision, but functions of some orphan subunits are just beginning to be established. Functional roles for postsynaptic AChRs in muscle are well known, but in neurons the post-, peri-, extra-, and presynaptic roles of AChRs are just being revealed. Pathogenic roles of AChRs are being discovered in many diseases involving mechanisms ranging from mutations, to autoimmune responses, to the unknown; involving cell types ranging from muscles, to neurons, to keratinocytes; and involving signs and symptoms ranging from muscle weakness to epilepsy, to neurodegenerative disease, to psychiatric disease, to nicotine addiction. Awareness of AChR involvement in some of these diseases has provoked new interests in development of therapeutic agonists for specific AChR subtypes and the use of expressed cloned AChR subunits as possible immunotherapeutic agents. Highlights of recent developments in these areas will be briefly reviewed.

Index Entries: Nicotinic acetylcholine receptors; nicotine; myasthenia gravis; Alzheimer's disease; Parkinson's disease; schizophrenia; epilepsy.

Introduction

Nicotinic acetylcholine receptors (AChRs) are an archetype of transmitter-gated ion channel from a gene super family of homologous receptors for GABA, glycine, and serotonin (Betz, 1990; Barnard, 1992). Their electrophysiological properties were initially best characterized in skeletal muscles, and their structural properties were initially best characterized using AChRs from the homologous electric organ tissue of *Torpedo* rays (Changeux, 1990; Karlin and Akabas, 1995; Lindstrom, 1996). Functional and structural characterization of neuronal AChRs developed later because of their lower concentrations in more heterogeneous tissues. Now the subunits of most or all AChR subunits have been cloned and

expressed. Although we now know vastly more about both the structure and functional properties of neuronal AChRs than just a few years ago, we still know more about the properties of expressed cloned subunit combinations than we do about the diverse physiological roles of the many potential subtypes of these AChRs. It is clear that many neuronal AChRs do not appear to function in the classic postsynaptic, directly excitatory, high safety factor process characteristic of neuromuscular transmission. Instead, neuronal AChRs are being found not only in postsynaptic, but also in pre-, peri-, and extrasynaptic locations where they can influence transmission by modulating release of many transmitters, respond to ACh at a distance from the sites of release, trigger inhibitory responses, alter neuronal process growth during development or plasticity, or perform roles that remain to be discovered (Lipton and Kater, 1989; Ullian and Sargent, 1995; Horch and Sargent, 1995, 1996; Quick, 1995; Role and Berg, 1996; Zhang et al., 1996; Wonnacott, 1977).

AChRs are associated with a growing list of diseases. The disease first and best characterized with the direct involvement of AChRs is autoimmune myasthenia gravis, in which an antibody-mediated autoimmune response to muscle AChRs impairs neuromuscular transmission (Lindstrom et al., 1988). Autoimmune responses to muscle AChRs have now also been implicated in an unusual paralytic fetal disease (Vincent et al., 1995). Recently, there has been dramatic progress in characterizing congenital myasthenia gravis caused by AChR mutations (Engel et al., 1997). Studies of these patients and of expressed cloned AChRs with their mutations have revealed more than gene knockout mice (Gomez et al., 1996) have about the structure and function of these AChRs. A mutation in a neuronal AChR has recently accounted for the first example of a mutation causing a specific form of epilepsy (Steinlein et al., 1995). Other diseases caused by mutations in neuronal AChRs will probably be found in the future, and these will help reveal the functional roles of the AChRs involved. It has been suggested that a genetic defect in a type of neuronal AChR may be among the factors predisposing to schizophrenia (Freedman et al., 1997). Substantial loss of neuronal AChRs is found in both of the major neurodegenerative disorders, Alzheimer's disease, and Parkinson's disease (Whitehouse et al., 1988; Perry et al., 1995). This loss is no doubt secondary to other pathological processes, but it has still stirred interest in development of subtype-specific neuronal AChR agonists for potential symptomatic therapy in these diseases (Arneric et al., 1994; Decker et al., 1994; Bjugstad et al., 1996; Sacaan et al., 1997; Menzaghi et al., 1997a,b). Nicotine itself has been reported to be useful in treating Tourette's disease (Silver et al., 1995). It has been suspected that volatile anesthetics act by potentiating the inhibitory responses of $GABA_A$ receptors, but recently it has been found that a subtype of neuronal AChR is blocked by very low concentrations of these anesthetics, suggesting that neuronal AChRs may be involved in some component of anesthesia or its side effects (Evers et al., 1997; Flood et al., 1997; Violet et al., 1997; Zhang et al., 1997). Ethanol has been suspected to produce its neurological effects through altering membrane lipids (Deitrich et al., 1989) and indirectly or directly altering ion channels (Weight et al., 1993). Recently, evidence was provided for a potent effect of ethanol on some nicotinic AChR subtypes, suggesting that AChRs may be involved in the initial behavioral effects of ethanol (Nagata et al., 1996; Yu et al., 1996). By far the largest medical impact of AChRs is the role of neuronal AChR subtypes as the primary mediators of nicotine's ability to cause addiction to tobacco (Koop, 1988; Peto et al., 1992; Benowitz, 1996). Nicotine also has some important effects on blood pressure and heart rate, presumably mediated by neuronal AChRs in tobacco users (Benowitz, 1996). In some circumstances, nicotine may act through neuronal AChRs as a mitogenic agent perhaps involved in promoting some forms of lung cancer (Schuller et al., 1995). Small cell carcinomas of the lung that express voltage-sensitive calcium channels are thought to provide the immunogen that stimulates the antibody-mediated

autoimmune response that impairs ACh release in the paraneoplastic disease Lambert Eaton Myasthenic syndrome (Vincent et al., 1989; Lang et al., in press). Such carcinomas also express neuronal AChRs (Tarroni et al., 1992; Sher and Clementi, in press). Recently, an example of muscle AChR expression was reported on such a carcinoma from a myasthenia gravis patient and proposed as the initiator of a paraneoplastic response in this patient (Lennon et al., in press).

This review will first examine the structural and functional features of AChR subtypes and functional roles that have been proposed for them. Then some of the medical roles of these AChRs will be examined. This is intended to provide a uniting perspective on all of these topics rather a comprehensive review of any of them.

AChR Structure and Function

AChRs are thought to be formed from five homologous subunits oriented like barrel staves around a central cation channel (Fig. 1). cDNA sequences for 16 kinds of AChR subunits have been determined in several species (McGehee and Role, 1995; Lindstrom, 1996). This includes α1–α9, β1–β4, γ, δ, and ε. Initially the subunits of purified electric organ and muscle AChRs were named α, β, γ, and δ in order of increasing apparent molecular weights from 40,000 to 65,000. Then α1 subunits were affinity labeled by the competitive antagonist MBTA which, after reduction of a disulfide bond between cysteines a192 and 193, covalently reacts at these amino acids to block the ACh-binding site (Kao and Karlin, 1986). Other homologous cDNAs that contained this cysteine pair were thus termed α2, 3... in order of their discovery. Putative neuronal AChR subunits lacking this cysteine pair are now usually referred to as β2–β4. The adult form of muscle AChRs has ε subunits substituted for γ subunits of the fetal form (Witzemann et al., 1990).

All subunits of AChRs and of other receptors in this superfamily have a characteristic pattern of structural features extending from their N-terminus to their C-terminus (Fig. 2) (Lindstrom, 1996). First, all have an N-terminal signal sequence that is cleaved during translation after the mature N-terminus crosses the membrane. Second, the N-terminal 210–220 amino acids form a large extracellular domain. This contains a disulfide-linked loop homologous to the one between cysteines 128 and 142 of α1 subunits and one or more *N*-glycosylation sites. The third feature shared by all of these subunits is a set of three closely spaced transmembrane domains immediately following the large extracellular domain that are termed M1–M3. There is good evidence that M1 and M2 are α helical (Karlin and Akabas, 1995), and it is presumed that the other transmembrane domains are also α helical. Each is 20–30 amino acids long. M1 is thought to extend to the cytoplasmic surface where a 6 or 8 amino acid linker connects it to M2. M2 is thought to cross back to the extracellular surface where a 6 or 8 amino acid linker joins it to M3, which then reaches back across the membrane to the cytoplasmic surface. The fourth common feature is a large cytoplasmic domain ranging in length from approx 91 amino acids in α5 subunits to approx 203 amino acids in α4 subunits. The next common feature is a fourth transmembrane domain, termed M4, which extends back to the extracellular surface. The final common structural feature is a short extracellular C-terminal domain of approx 4–28 amino acids.

Consideration of the evolution of AChR subunits provides an instructive perspective on their properties (LeNovere and Changeux, 1995). On the basis of sequence homologies, and the fact that α7, α8, and α9 subunits can form functional homomers, it has been proposed that α7 subunits represent the most primordial form of this family, and that the other subunits evolved through gene duplication and subsequent divergence. The muscle α1 subunit is quite a conserved sequence, showing 80% amino acid sequence identity between the electric organ of the marine elasmobranch *Torpedo* and skeletal muscle of humans, whose last common ancestor with *Torpedo* evolved more than 400 million yr ago (Noda et al.,

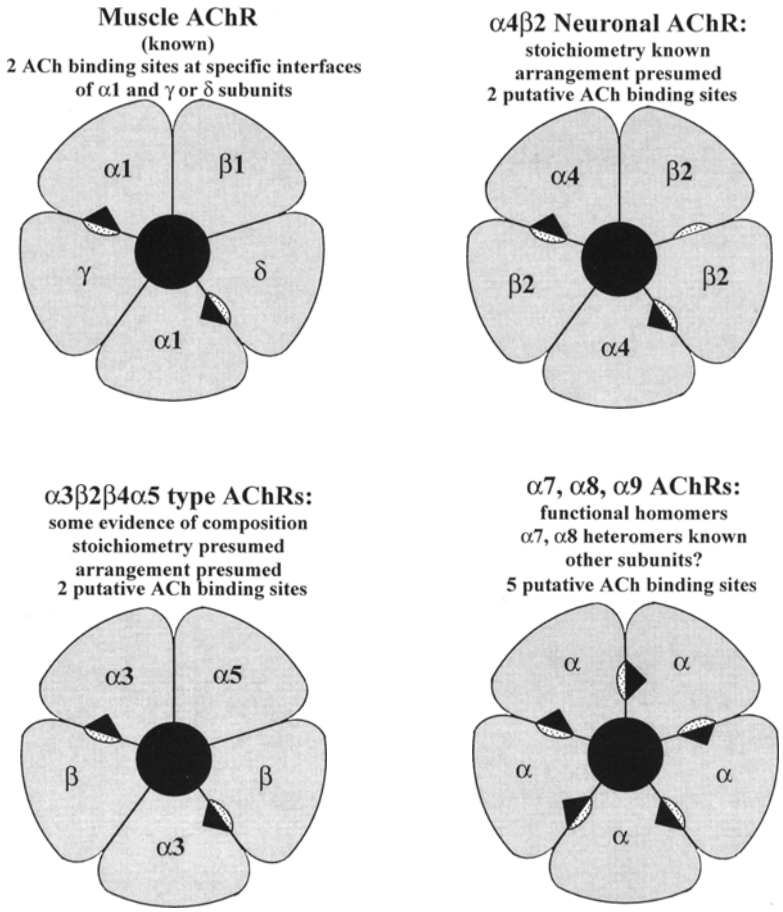

Fig. 1. Putative subunit arrangements around the central cation channel of some major AChR types. Reproduced from Wang et al. (1996).

1983). α1–α6 together with β2–β4 form a group of related sequences, as do the muscle structural subunits β1, γ, δ, and ε (LeNovere and Changeux, 1995). Several pairs of subunits are closely related in sequence, which is instructive in considering their functions. The ε and γ subunits of muscle AChRs are quite similar in sequence, consistent with the ability of ε to substitute for γ during the maturation from fetal to adult muscle AChR (Weitzmann et al., 1990). The β2 and β4 subunits are also very close in sequence, consistent with their ability to substitute for one another in forming neuronal AChRs when paired with α2, α3, or α4 subunits (Papke, 1993). β2 and β4 subunits each typically confer somewhat different pharmacological properties when expressed in combination with a subunits, consistent with the idea that β2 and β4 contribute different contact amino acids to an ACh-binding site formed at the interface between α2, α3, α4 or α6 subunits and β2 or β4 subunits (Papke, 1993). Similarly, α2 and α4 are closely related. It is especially interesting that α3 and α6 have very similar sequences (LeNovere and Changeux, 1995), because until recently α6 was an orphan subunit that had not been shown to form functional AChRs. Now α6, like α3, has been found to form functional AChRs in combination with β2 or β4 subunits (Gerzanich et al., 1997; Gerzanich, Kuryatov and Lindstrom, unpublished results), but these differ pharmacologically from those formed in combination with α3 subunits. For example, on α6β4 AChRs,

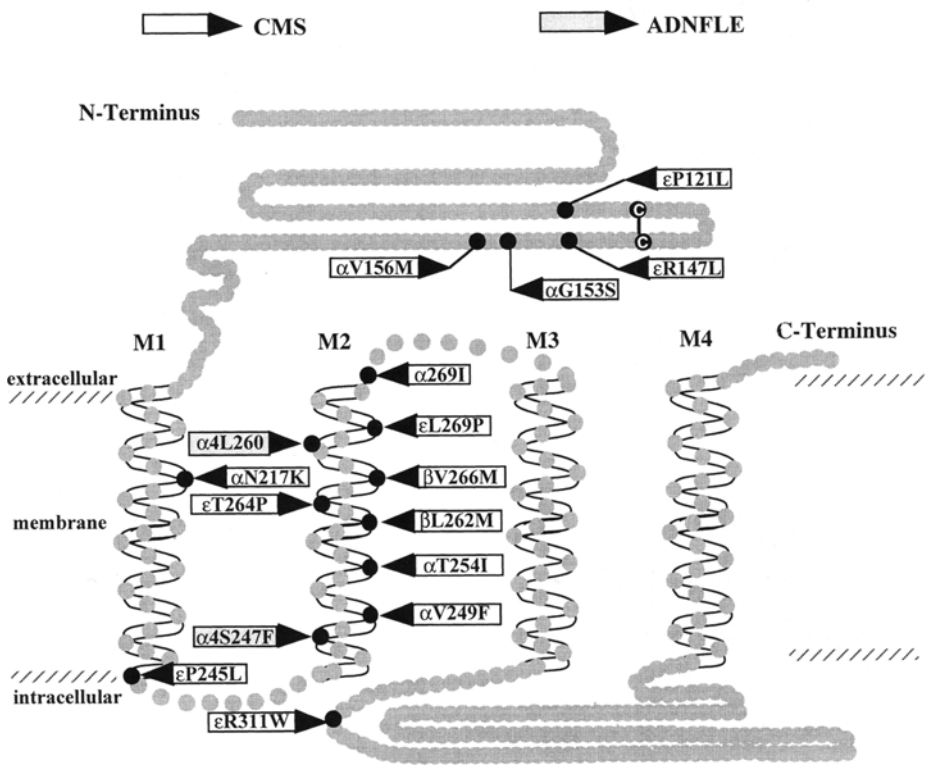

Fig. 2. Transmembrane orientation of a generic AChR subunit indicating approximate sites of mutations in congenital myasthenic syndromes (CMS) and autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE).

nicotine is only an 18% partial agonist (Gerzanich et al., 1997). The close similarity in sequence between α3 and α6 subunits may also have caused misidentification of α6 subunits as α3 subunits in some brain nuclei such as the substantia nigra (that is pathologically critical in Parkinson's disease) when insufficiently specific α3 subunit probes were used in early *in situ* hybridization studies (LeNovere et al., 1996). A final interesting pair of closely related subunits are β3 and α5. Until recently, both were orphan subunits. Both β3 and α5 are unable to form functional AChRs when expressed alone or as pairs with other subunits. First it was found that α5 could be immunologically identified as part of ganglionic AChRs that also contained α3 and β4 subunits (Conroy et al., 1992). Then it was found that cloned α5 could assemble efficiently when coexpressed with α3β2, α3β4, or α4β2 combinations (Wang et al., 1996; Ramirez-Latorre et al., 1996). In combination with human α3 and β2 or β4 subunits, α5 increased the desensitization rate, and with the α3β2 combination α5 altered EC_{50} values for ACh and nicotine and increased the efficacy of nicotine from 50–100% (Wang et al., 1996). It was proposed that the presence of a 192, 193 cysteine pair in α5 is misleading, that it really cannot form an ACh-binding site in paired combination with β subunits, and that when it is part of AChRs as a third subunit it occupies a position equivalent to β1 subunits of muscle AChRs so that it interfaces with the surfaces of adjacent subunits that are incapable of forming ACh-binding sites (Fig. 1) (Wang et al., 1996). Recently, β3 subunits were similarly immunoisolated as part of rat striatum and cerebellum AChRs containing α4, β2, and β4 subunits and also shown to combine with these subunits to form ^{3}H-ACh binding AChRs when this mixture of subunit cDNAs were coexpressed (Forsayeth and Kobrin, 1997). It

seems likely that β3 occupies the same position in AChRs that α5 would occupy.

Subunit stoichiometry and arrangement is best known in the case of AChRs from *Torpedo* electric organ (Fig. 1) (Karlin and Akabas, 1995). These AChRs consist of two α1 subunits and one each of β1, γ, and δ (Reynolds and Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). Cloned α7, α8, and α9 expressed in *Xenopus* oocytes can form functional homomers of a size consistent with being pentamers (Anand et al., 1993; Elgoyhen et al., 1994). In chick brain and retina α7 and α8 can also be found together as part of heteromers (Keyser et al., 1993). The stoichiometry of cloned α4β2 AChRs expressed in *Xenopus* oocytes consists of two α4 subunits and three β2 subunits (Anand et al., 1991; Cooper et al., 1991). It is supposed that pairwise expression of α2–6 subunits with β2 or β4 subunits results in similar stoichiometries. Neuronal AChRs containing four kinds of subunits have been immunoisolated (e.g., α3β2β4α5 [Conroy and Berg, 1995] or α4β2β4β3 [Forsayeth and Kobrin, 1997]). It is suspected that the arrangement of subunits in these neuronal AChRs is homologous to that in muscle AChRs.

Only in the case of AChRs from electric organ and muscle is there detailed evidence for the arrangement of subunits around the central ion channel. Morphological evidence for a symmetric arrangement of subunits around a central ion channel is provided by the pentagonal arrangement of rod-like subunits around a central channel determined by diffraction analysis of electron micrographs of two dimensional crystalline arrays of electric organ AChRs (Unwin, 1993, 1995). Affinity labeling of homologous amino acids in the M2 region of all subunits by channel-blocking ligands provides evidence that the M2 domain of each subunit contributes to the lining of the channel (Hucho et al., 1986; Karlin and Akabas, 1995). Affinity labeling experiments indicate that ACh-binding sites occur at particular interfaces between α1 and γ, δ, or ε subunits (Pederson and Cohen, 1990; Czajkowski and Karlin, 1995; Martin et al., 1996). Expression of α1γ, α1δ, or α1ε subunit pairs results in the formation of ACh-binding sites characteristic of one or the other of the two types of sites characteristic of intact AChRs (Blount and Merlie, 1989; Sine, 1993; Fu and Sine, 1994, 1996; Sine et al., 1995; Wang et al., 1996). Combined electron microscopy and crosslinking experiments argue that the order of subunits around the channel is α1, γ, α1, δ, β (Karlin et al., 1983). In neuronal AChRs it is thought that similar constraints result in two ACh-binding sites at corresponding subunit interfaces in heteromeric AChRs and perhaps five ACh-binding sites in homomeric AChRs, as depicted in Fig. 1.

The ACh-binding sites appear to involve contact amino acids from several parts of the extracellular domain of α1 subunits as well as some negatively charged amino acids from the γ, δ, or ε subunits whose electrostatic interactions, along with π electron interactions from aromatic amino acids, may contribute to binding of the positively charged quaternary amine of ACh (Galzi et al., 1990, 1991; Galzi and Changeux, 1994; O'Leary et al., 1994; Czajkowski and Karlin, 1995; Martin et al., 1996). The location of ACh-binding sites at the interfaces between subunits may facilitate small cooperative motions of subunits to produce the conformational change that opens the cation channel when agonists are bound to both ACh-binding sites. A combination of affinity labeling and mutagenesis experiments has suggested that amino acids from three parts of the extracellular domain of Torpedo α1 (around Y93, around W149, and the region including Y190, C192, C193, and Y198) contribute to the ACh binding site as do two parts of the adjacent subunit (γ W55 and γ D174 or δ W57 and δ D180) (Galzi et al., 1990, 1991; Galzi and Changeux, 1994; O'Leary et al., 1994; Czajkowski and Karlin, 1995; Martin et al., 1996). Homologous amino acids probably contribute to the ACh-binding sites of neuronal AChRs (Galzi et al., 1991).

The SCAM method (substituted cysteine accessibility method) has proven especially valuable in defining the structure of the cation channel (Akabas et al., 1992; 1994; Akabas and Karlin,

1995; Karlin and Akabas, 1995). These experiments suggest that M1 and M2 are especially important. M1 is adjacent to a major part of the ACh-binding site, and thus might be especially well positioned to provide leverage between ACh binding and opening the channel gate. M1 is thought to be α helical and partially exposed in the channel, with this exposure changing during gating or desensitization (Akabas and Karlin, 1995; Karlin and Akabas, 1995). The loop between M1 and M2 is thought to contribute to the structure of the gate (Karlin and Akabas, 1995). M2 is thought to be an amphipathic α helix, interrupted around L250 to S252 in the resting state, with its hydrophilic amino acids in the channel lumen extending to E241 at nearly the cytoplasmic surface in the closed state of α1 subunits, and with activation causing a change to a fully α helical conformation (Akabas et al., 1994; Karlin and Akabas, 1995). The basic homology in channel architecture between receptors in the superfamily was elegantly proven by the demonstration that only three amino acids characteristic of the M2 domains of the anion selective channels of $GABA_A$ and glycine receptors need to be substituted into the M2 region of α7 AChRs to convert the selectivity of their channels to anions (Galzi et al., 1992).

The three dimensional structure of *Torpedo* AChRs has been determined to a resolution of 9Å by diffraction studies of two-dimensional crystalline arrays (Unwin, 1993, 1995). At this resolution, α helices cannot be clearly resolved, and all AChR subtypes would probably look fairly similar. Viewed from the extracellular surface, the AChR is pentagonal, about 80Å across, with about 25Å thick walls surrounding a 25Å diameter channel lumen. Viewed from the side, the AChR is roughly cylindrical, with about 65Å extending on the extracellular surface, 40Å crossing the membrane, and about 15Å on the cytoplasmic surface.

The AChR family tree can be generally thought of as having three branches: muscle type AChRs, neuronal AChRs that do not bind the snake venom toxin α bungarotoxin (αBgt), and neuronal AChRs that do bind αBgt.

Muscle type AChRs include both AChRs from electric organs and skeletal muscle (Changeux, 1990; Karlin and Akabas, 1995). These bind αBgt at their ACh binding sites. There are only two subtypes, a fetal $\alpha1\beta1\gamma\delta$ form and an adult $\alpha1\beta1\varepsilon\delta$ form. At mature endplates approx 2×10^7 AChRs are located at the tips of folds in the muscle postsynaptic membrane directly across from active zones where ACh is released from the motor nerve ending (Engel, 1994). Their functional role is to ensure neuromuscular transmission by amplifying the small motor nerve current sufficiently to trigger an action potential in the muscle. Excesses of both ACh and AChRs ensure a high safety factor (on the order of 10-fold) for neuromuscular transmission (Magelby, 1994).

A second branch of the AChR family is neuronal AChRs formed from combinations of α2, α3, α4, or α6 subunits with β2 or β4 subunits, sometimes including α5 or β3 subunits in addition (Sargent, 1993; McGehee and Role, 1995; Lindstrom, 1996). These do not bind αBgt. A huge number of permutations and combinations of subtypes are possible, but a few predominate. α4β2 AChRs account for >90% of the high affinity nicotine-binding sites in brain (Whiting et al., 1988; Flores et al., 1992). α3 AChRs, α6 AChRs and others of this group are thought to be present in smaller amounts in more limited regions of the brain. Although glutamate receptors are by far the predominant excitatory transmitter-gated ion channels in the brain, there are smaller amounts of AChRs throughout the brain (Clarke et al., 1985; Swanson et al., 1987; Del Toro et al., 1994). Demonstration of classical postsynaptic AChRs in the brain has been difficult, but there is ample electrophysiological and synaptosome release evidence for presynaptic AChRs that can modulate the release of many transmitters (Gray et al., 1996; Role and Berg, 1996; Lena and Changeux, 1997; Wonnacott, 1997). In theory, only a small number of presynaptic AChRs would be required to increase transmitter release by increasing the calcium concentration in the small nerve ending volume. All of the neuronal AChR subtypes examined are more calcium permeable than are the channels of muscle AChRs (Lindstrom et al., 1995; Rathouz

et al., 1995). In peripheral ganglia, such as ciliary ganglia, α3 AChRs are located postsynaptically as well as perisynaptically (Horch and Sargent, 1995, 1996). These neurons contain approx 80% of their α3 AChRs with the subunit composition α3β4α5 and approx 20% with the subunit composition α3β2β4α5 (Conroy and Berg, 1995). These neurons also express α7 AChRs. Coexpression of both multiple α3 AChR subtypes and α7 AChRs is typical of adrenal medullary chromaffin cells (Garcia-Guzman et al., 1995), pheochromocytomas (Chan and Quick, 1993), and several neuroblastoma lines that have been studied (Tarroni et al., 1992; Gotti et al., 1995; Peng et al., 1997).

The third branch of the AChR family tree includes AChRs composed of α7, α8, or α9 subunits (Schoepfer et al., 1990; Couturier et al., 1990; Elgoyhen et al., 1994). These can function as homomeric AChRs (Couturier et al., 1990; Seguela et al., 1993; Gerzanich et al., 1994; Peng et al., 1994; Elgoyhen et al., 1994). In chickens, α7α8 heteromers have also been found (Keyser et al., 1993). Their functional activity was initially not detected in neurons, but is now clearly recognized (Albuquerque et al., 1996; Zhang et al., 1996; Gray et al., 1996). These AChRs are antagonized by αBgt. Two other characteristic features of these AChRs are a very rapid rate of desensitization and a high level of selectivity for calcium approaching that of the NMDA type of glutamate receptor (Seguela et al., 1993; Gerzanich et al., 1994; Elgoyhen et al., 1994; Albuquerque et al., 1996). Like NMDA receptors, α7 AChRs show voltage sensitive magnesium ion block of the ion channel; but, unlike NMDA receptors, α7 AChRs are blocked when the membrane depolarizes (Bertrand et al., 1995; Albuquerque et al., 1996). Rapid desensitization should limit the ability of such AChRs to respond to sustained intense stimulation. High calcium permeability should permit calcium ions to act as second messengers to affect many cellular processes. Rapid desensitization for a long time prevented the discovery that α7 AChRs were functional ACh-gated cation channels. In brain, there are about as many α7 AChRs as α4β2 AChRs (Clarke et al., 1985; Whiting and Lindstrom, 1988), and in ciliary ganglia α7 AChRs far outnumber α3 AChRs (Vernalis et al., 1993). α8 has been found only in chickens (Schoepfer et al., 1990; Keyser et al., 1993). α9 is found only in limited areas of the rat nervous system (Elgoyhen et al., 1994). α7 AChRs in ciliary ganglia are located perisynaptically (Horch and Sargent, 1995). It had long been thought that they played no role in ganglionic transmission because they were not immediately postsynaptic and because αBgt did not block transmission. Now it has been realized that there is actually a high safety factor for ganglionic transmission, and that it can be mediated by α7 AChRs if the α3 AChRs are blocked (Zhang et al., 1996). This raised the interesting question of at what distances from the site of release ACh might act for transmission or trophic effects in parts of the brain. Postsynaptic α9 AChRs in cochlear hair cells have the interesting property that their fast-acting excitatory activity triggers a long-lasting inhibitory response through calcium-activated potassium channels (Fuchs, 1996). This raises the interesting question of what other unusual synaptic and nonsynaptic signaling mechanisms and gene regulation might be mediated through α7 AChR-regulated calcium ion flow in the brain. Presynaptic α7 AChRs in the hippocampus have been shown to regulate release of glutamate (Gray et al., 1996). α7 AChRs have also been implicated in regulating neuronal process outgrowth (Pugh and Berg, 1994; Quick, 1995), providing protection against glutamate excitotoxicity (Martin et al., 1994; Donnelly-Roberts et al., 1994), and regulating mitogenic activity in some carcinomas (Codignola et al., 1994; Schuller et al., 1995; Fucile et al., 1997). Overall, the functional roles nicotinic AChRs throughout the brain remain to be discovered. α7 AChRs have also recently been found at early developmental times in muscle and tendon (Romano et al., 1997a,b). Here too, their functional roles remain unknown.

Roles of AChRs in Diseases

Muscle AChRs

Autoimmune Myasthenia Gravis

Autoimmune myasthenia gravis (MG) is characterized by skeletal muscle weakness and fatigability caused by an antibody-mediated autoimmune response to muscle AChRs (Lindstrom et al., 1988). Immunization of animals with AChRs purified from electric organs or muscle causes experimental autoimmune MG (EAMG) (Lindstrom et al., 1988). Autoantibodies to AChR impair neuromuscular transmission in MG by three mechanisms. First, crosslinking of AChRs by antibodies increases their rate of internalization and lysosomal destruction, resulting in a net loss of AChRs by a process termed antigenic modulation. Second, binding of antibodies to the extracellular surface of AChRs permits binding of complement, resulting in focal lysis of the postsynaptic membrane that both causes loss of AChRs and disrupts the morphology of the postsynaptic membrane so that AChRs at the tips of folds in this membrane are no longer tightly opposed to active zones of ACh release from the presynaptic membrane. Third, in some patients, autoantibodies directly impair AChR function, but overall this effect appears to contribute less to lowering the safety factor for neuromuscular transmission than does loss of AChRs and disruption of the postsynaptic membrane.

Half or more of the autoantibodies in MG and EAMG are directed at the main immunogenic region (MIR) (Tzartos et al., 1982, 1991). Monoclonal antibodies to the MIR can cause antigenic modulation and complement-mediated damage (Tzartos et al., 1987, 1991). The MIR includes as critical amino acids α68 and α71 (Saedi et al., 1990; Tzartos et al., 1991). The MIR is located at the extracellular tip of α1 subunits and is angled away from the central axis of the AChR molecule in such a way that a single MAb cannot crosslink the two α1 subunits in an AChR (Conti-Tronconi et al., 1981; Beroukin and Unwin, 1995). The prominent extracellular location of the MIR facilitates antibody binding and complement fixation. The orientation of the MIR on α1 subunits expressed twice in each monomer facilitates the crosslinking of AChRs, which causes antigenic modulation. The bivalency, epitope sequence, prominent extracellular location, and crosslink-promoting orientation of the MIR probably also contribute to its immunogenicity. A similar MIR amino acid sequence is found on α1, α3, α5, and β3 subunits (Lindstrom et al., in press). MAbs to the MIR have been shown to bind to human α1, α3, and α5 subunits (Wang et al., 1996; Kuryatov et al., in press). It is unknown whether MG patient autoantibodies crossreact with AChRs containing α3 and α5 subunits. If this were to occur, it might impair transmission in adrenal medulla and peripheral ganglia, for example, where AChRs that react with MAbs to the MIR are found (Conroy and Berg, 1995; Horch and Sargent, 1996; Wenger et al., 1997). No antibodies in MG patient sera were found to human brain AChR subtypes that lack subunits with an MIR (Whiting et al., 1987). Specifically, no antibodies were detected to either high-affinity nicotine binding AChRs (i.e., α4β2 AChRs) or high affinity aBgt binding AChRs (i.e., α7 AChRs).

A particularly instructive example of disease caused by autoimmune response to muscle AChRs was recently reported (Vincent et al., 1995). Arthrogryposis multiplex congenita (AMC) is characterized by multiple joint contractures that can cause fetal death. A woman was found who, after a normal birth, had six subsequent pregnancies affected. She had no signs of MG, but was found to contain antibodies which potently inhibited the function of fetal but not adult muscle AChRs. Her serum blocked a maximum of half of the binding of αBgt to fetal AChRs. Evidently, antibodies to the part of γ subunits that contribute to forming one of the two ACh-binding sites are responsible for AMC in this case. Inhibiting function of fetal α1β1γδ AChRs initially present over the entire surface of muscle cells probably inhibits the formation of neuromuscular junctions, resulting in fetal muscle wasting and contracture while sparing the α1β1εδ AChRs at the mature neuromuscular junctions of the mother.

What initiates the autoimmune response to AChRs in MG is unknown. The occurrence of thymoma in approx 12% of MG patients makes MG in some patients a cancer-related disease (Newsome-Davis and Vincent, 1991). These thymoma cells do not in general express muscle AChRs, although muscle AChR subunit cDNAs have been cloned from thymomas and neuronal AChR α3, β4, and α5 subunits have been detected in thymus (Hara et al., 1993; Mihovilovic and Roses, 1993; Gattenlohner et al., 1994). The paraneoplastic disease Lambert Eaton Myasthenic syndrome (LEMS) offers some instructive parallels (Vincent et al., 1989). The muscular weakness characteristic of LEMS is caused by autoantibodies to voltage-gated calcium channels and related components of the active zones that regulate ACh release from motor nerve endings. Most of these patients are smokers who are found, often long after diagnosis of LEMS, to have small-cell lung carcinomas. These carcinomas express voltage-gated calcium channels. It is thought that LEMS is a side effect of an immune response that is holding these slow-growing tumor cells in check. Small-cell lung carcinomas have also been found that express neuronal AChRs (Tarroni et al., 1992; Codignola et al., 1994). Recently a patient was reported with MG who had a small cell carcinoma of the lung that expressed muscle-type AChRs (Lennon et al., in press). Perhaps in this patient the immune response to the tumor also caused MG. Perhaps in some other MG patients there are other unrecognized slow-growing tumors held in check by an autoimmune response to AChRs that also is responsible for their MG.

The ideal therapy for MG would specifically suppress only the autoimmune response to muscle AChRs. Current therapy (Drachman, 1994) includes the use of inhibitors of ACh esterase to provide symptomatic relief by increasing the concentration and duration of ACh in the neuromuscular junction to compensate for loss and disruption of functional AChRs. It also includes use of steroid and cytotoxic drugs to nonspecifically suppress the immune response. Chronic treatment with prednisone is associated with many side effects including Cushing's syndrome. Cytotoxic drugs such as azathioprine risk nonspecific killing of many types of rapidly dividing cells. An approach to antigen-specific therapy that has recently received a lot of attention in studies of EAMG is oral tolerance (Wang et al., 1993a,b, 1994, 1995; Okamura et al., 1994; Ma et al., 1995, 1996; Drachman et al., 1996; Lindstrom et al., in press). Feeding an antigen can suppress the immune response to it through mechanisms intended to prevent unproductive immune responses to food components (Weiner, 1997). Nasal application of antigens can be even more potent in suppressing immune responses (Weiner, 1997). Oral or nasal application of purified *Torpedo* electric organ AChR is effective at suppressing EAMG-induced by subsequent immunization with *Torpedo* AChR (Wang et al., 1993a,b, 1994, 1995; Okamura et al., 1994; Ma et al., 1995, 1996). Treating ongoing EAMG by mucosal application of purified *Torpedo* AChR has been more problematic (Drachman et al., 1996). Native AChR and its conformation-dependent MIR are highly immunogenic and antibodies to the MIR are pathogenic (Lindstrom et al., 1988; Tzartos et al., 1991). It is thought that mucosal tolerance is mediated through T-lymphocytes (Weiner et al., 1997). T-lymphocytes do not recognize native protein antigens, but instead recognize small peptide fragments of these proteins after digestion by antigen-presenting cells and presentation on their surface bound to MHC class II proteins. We have obtained encouraging preliminary results in both preventing and treating EAMG by oral and nasal treatment with bacterially expressed human muscle AChR proteins (Lindstrom et al., in press). The idea is that bacterial expression provides large amounts of muscle AChR subunits, and that these subunits retain T-cell epitopes that can act to promote mucosal tolerance, but lack conformation-dependent epitopes like the MIR that are especially potent in provoking pathologically significant autoantibodies.

Congenital Myasthenic Syndromes

Congenital myasthenic syndromes (CMS) can be caused by mutations in many processes that impair neuromuscular transmission (Engel et al., 1977). These include defects in ACh packaging and release, ACh esterase, and muscle AChRs among others. A ground swell of progress has recently been made in elegant characterization of a series of mutations in AChR subunits that are responsible for various forms of CMS (Engel et al., 1993, 1996 a,b; 1997; Ohno et al., 1995, 1996, 1997; Sine et al., 1997). More than 60 mutations have been recognized by Andrew Engel and his coworkers (personal communication) and many have been published in detail (Engel et al., 1993, 1996a,b, 1997; Ohno et al., 1995, 1996, 1997; Sine et al., 1997). These combined studies of patient symptoms, endplate electrophysiology and morphology, as well as electrophysiological and biochemical analysis of expressed cloned mutant AChRs, reveal our dependence on the relative perfection of the evolution of our muscle AChRs, and are more informative about the human effects of these mutations than transgenic mice would be. Each mutation has interesting subtle differences in phenotype. There is a large safety factor for neuromuscular transmission, consequently some people may function with only moderate impairment, despite some mutations until challenged by AChR antagonist muscle relaxants given during surgery (Engel et al., 1996a). Others survive potentially lethal truncation mutations of the ε subunits of adult AChR forms by expression of γ subunits of fetal AChRs (Engel et al., 1996a; Ohno et al., 1995, 1997). Reduced AChR activity because of reduced affinity for ACh (caused by an extracellular εP121L mutation [Ohno et al., 1996]) reduces channel open time and length of bursts of openings (termed a "fast channel syndrome") as does an εR311W mutation at the beginning of the large cytoplasmic domain (Fig. 2) (Ohno et al., 1997). Of course, these mutations impair transmission (Ohno et al., 1996). These mutations are also instructive about the sequences associated with particular functional properties. Increased AChR function results in pleotropic excitotoxic effects that can result in impaired transmission (Engel et al., 1996b, 1997). Excessive calcium ion influx can activate proteases and other processes that result in disrupted postsynaptic morphology and reduced numbers of AChRs. Sustained depolarization from hyperactive AChRs can also result in inactivation of perijunctional sodium channels. One cause of increased AChR function is an increase in affinity for ACh, resulting in prolonged bursts of channel openings (Sine et al., 1995). This is caused by an αG153S mutation in the extracellular domain of α1 subunits near known contact amino acids for the ACh binding site. Patients with the αG153S mutation that causes slowed dissociation of ACh are less severely effected than are those afflicted with many of the other mutations. The nearby extracellular domain mutation αV156M may stabilize the open state (Croxen et al., 1997). Spontaneous channel openings have been found to be caused by the mutations βV266M and εL269F (Engel et al., 1996a,b; Ohno et al., 1995; Milone et al., 1996). Excitotoxic prolonged ("slow") channel openings have been found to result from mutations in several parts of the AChR. Most slow channel mutations have been found in the M2 transmembrane domain thought to provide much of the channel lining and perhaps part of the gating mechanism. These M2 mutations include the mutations αV249F, αT254I, βL262M, βV266M, εT264P, and εL269F (Gomez et al., 1995, 1996; Ohno et al., 1995; Engel et al., 1996a; Croxen et al., 1997). These M2 mutations may have multiple effects, for example αV249F increases apparent affinity for ACh and enhances desensitization (Milone et al., 1996), but βV266M does not (Engel et al., 1996b). Slow channel syndromes also result from mutations in the M1 transmembrane domain that may link the ACh binding site and M2 channel lining domain as well as provides some of the channel lining. These M1 mutations include αN217K and εP245L (Engel et al., 1996a; Ohno et al., 1997). The εP245L mutation is at the cytoplasmic end of M1 near the M1–M2 loop

implicated in forming the channel gate (Karlin and Akabas, 1995). These M1 slow-channel mutations may have multiple effects, for example αN217K also enhances desensitization. A slow-channel syndrome has also been found to result from a less likely region, an αS269I mutation in the putative extracellular loop between M2 and M3 (Croxen et al., 1997). Mutations have been found that cause truncation of ε subunits (Engel et al., 1993a, 1996a; Ohno et al., 1997). It is thought that these are compensated for by expression of γ subunits. Mutations in parts of the extracellular domain of ε associated with subunit assembly (e.g., εR147L) cause reduced AChR expression that is partially compensated for by expression of γ subunits (Ohno et al., 1997). The εR311W mutation at the beginning of the large cytoplasmic domain after M3 also impairs assembly and is compensated for by γ (Ohno et al., 1997). Many of these mutations are autosomal recessive because of the large safety factor for neuromuscular transmission, yet the frequency of mutations is high enough that several patients have been found whose disease results from two recessive mutations (Engel et al., 1996a,b; Ohno et al., 1996, 1997).

The large number of mutations that have been found in muscle AChRs raises the question of how many mutations might be found in neuronal AChRs. Of course, with neuronal AChRs there are many problems. It is difficult to predict what symptoms to expect from decreased or increased function of the many neuronal AChR subtypes, because of their poorly understood normal functions. Also, the inaccessibility of most neuronal AChRs to biopsy precludes elegant patient studies like those on CMG. Nonetheless, mutations in a neuronal AChR have been recognized.

α4 Subunit Mutations Cause a Form of Epilepsy

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is a newly recognized form of epilepsy caused by mutations in α4 subunits (Steinlein et al., 1995, 1997). It was the first mutation shown to cause a form of epilepsy. In ADNFLE brief partial seizures occur during light sleep and are often misdiagnosed as nightmares (Scheffer et al., 1995). Most patients also have some secondary generalized seizures. In one mutation causing ADNFLE a highly conserved serine at position 247 of the M2 channel lining domain of α4 subunits is replaced by a phenylalanine (Fig. 2). Not surprisingly, channel function is impaired by this α4 S247F mutation in which a small hydrophylic hydroxyl group side chain of serine in the channel lumen is replaced by the large hydrophobic phenyl group of phenylalanine at a position thought to be near the gate at the cytoplasmic surface of the channel (Steinlein et al., 1995). Four mechanisms have been found to reduce the net flow of ions through mutant α4β2 AChRs: Ca^{2-} permeability is lost, channel open probability is reduced largely because of an increased rate of desensitization, channel open time is reduced, and channel conductance is reduced (Weiland et al., 1996; Kuryatov et al., in press). Two compensating mechanisms have also been identified: incorporation of α5 subunits repairs the deficit in Ca^{2+} conductance, and repeated activation produces a permanent conformation change resulting in a more activatable AChR (Kuryatov et al., in press). The net effect of the mutation is so potent that disease occurs even in heterozygotes.

A second α4 subunit mutation that causes ADNFLE is an insertion of a leucine after position 259 near the extracellular C-terminal end of M2 (Steinlein et al., 1997). This insertion may be relatively well tolerated because it follows a stretch of three other leucines and precedes an isoleucine. This mutation increases the potency of ACh 13-fold, resulting in an EC_{50} of 0.28 μ*M*, but as in the case of the α4 S247F mutation, the calcium permeability is reduced. Thus, the net effect of this mutation may also be a net loss of function.

Since epilepsy is characterized by excessive neuronal activation (Dichter, 1994), how could mutations that decrease AChR function cause

ADNFLE? High doses of nicotine can cause seizures, presumably through activation or desensitization of various neuronal AChR subtypes (Benowitz, 1996). Loss of α4β2 AChRs in β2 knockout mice does not cause seizures, although it does cause interesting changes in some learning behaviors (Picciotto et al., 1995). Neuronal AChRs are effective at promoting release of many transmitters, including GABA, from synaptosomes, brain slices, and electrophysiologically monitored neurons (Lena and Changeux, 1997; Wonnacott, 1997). It may be that presynaptic α4β2 AChRs that facilitate release of the inhibitory transmitters gamma amino butyric acid or glycine or postsynaptic α4β2 AChRs responsible for stimulating such inhibitory neurons trigger ADNFLE through reduced inhibition at transition periods between sleep and wakefulness.

Alzheimer's Disease

Alzheimer's disease (AD) is characterized behaviorally by loss of ability to learn, by memory and attention deficits, by anxiety, and by depression. AD is characterized neuropathologically by amyloid plaques and tangles accompanying extensive neurodegeneration (Hardy, 1997). Among the many changes in AD patients' brains is a large loss of high-affinity nicotine binding sites, suggesting that loss of some AChR subtypes occurs in the course of AD (Whitehouse et al., 1988). At one time there was a cholinergic hypothesis for the causation of AD, but now the primary effects seem most closely associated with processing of amyloid precursor protein to produce the amyloid characteristic of this disease (Hardy, 1997). However, some interest in nicotinic pharmacological approaches to treatment of Alzheimer's disease has continued because: there is no good therapy available, and the only approved therapy is the ACh esterase inhibitor tacrine (Hardy, 1997); nicotine has some beneficial effects on learning, memory, attention, anxiety, and cognition in AD patients (Sahakian et al., 1989; Newhouse et al., 1993; Benowitz, 1996; Everitt and Robbins, 1997); and there is AChR loss in AD (Whitehouse et al., 1988). This has led to interest in developing AChR subtype-specific agonists. In order to enter the brain, they must be tertiary amines. In order to prevent unwanted stimulation of muscle, these drugs are intended to have low affinity for muscle AChRs. In order to avoid gastrointestinal distress, nausea, hypothermia, increases in heart rate, and blood pressure among other nicotine effects thought to be associated primarily with stimulation of α3 AChRs in peripheral ganglia, these drugs are intended to have low affinity or efficacy on α3 AChRs. Abbott Pharmaceuticals investigated ABT418 as a selective agonist for α4β2 AChRs (Arneric et al., 1994; Decker et al., 1994). Taiho Pharmaceuticals is investigating the α7 selective agonist GTS21 (Martin et al., 1994; Meyer et al., 1994; Papke et al., 1994). AChR agonists would be expected to improve transmission by activating pre- or postsynaptic AChRs. However, predicting the effects of chronic treatment with nicotinic agonists is difficult because prolonged exposure to agonists can cause both reversible desensitization and irreversible inactivation of AChRs (Lukas, 1991; Collins and Marks, 1996; Dani and Heinemann, 1996; Hsu et al., 1996; Olale et al., in press). Chronic exposure to agonists can also induce upregulation in the amount of various AChR subtypes to various degrees (Schwartz and Kellar, 1983; Benwell et al., 1988; Flores et al., 1992; Marks et al., 1992; Peng et al., 1994, 1997). Another interesting effect of treatment of neurons with AChR agonists is protection from subsequent glutamate-induced excitotoxicity or from loss of nerve growth factor and serum growth factors (Akaike et al., 1994; Martin et al., 1994; Donnelly-Roberts et al., 1996; Shimohama et al., 1996; Messi et al., 1997). In the context of AD, an especially provacative neuroprotective effect reported for nicotine and the α7-selective agonist DMXB is complete protection of rat brain cortical neuron cultures from toxicity induced by the β amyloid protein characteristic of AD (Kihara et al., 1997). It was suggested that

these agonists might act through inhibition of nitric oxide production. Glutamate-induced excitotoxicity has been implicated in neuronal cell death associated with many neuronal disease processes including stroke (Meldrum and Gartwaite, 1990). The hypothesis is that excessive calcium ion entry resulting from excessive glutamate-receptor activation helps to trigger necrotic and apoptotic processes leading to cell death. Thus, it is interesting that activation of AChRs, which is associated with calcium influx (Vijayaraghaven, 1992; Seguela et al., 1993; Lindstrom et al., 1995), should be protective against subsequent larger calcium-mediated toxicity. The mechanisms involved are uncertain, although they are thought to involve entry of Ca^{2+}, and it has been reported that nicotine causes cytoprotection by permitting entry of Ca^{2+} that inhibits the formation of nitric oxide (Shimohama et al., 1996). Two possible explanations for why AChR activation is protective against subsequent glutamate excitotoxicity are: a small amount of calcium influx through AChRs triggers processes that are protective or desensitizes processes that are damaging when larger amounts of calcium enters later, or calcium influx through AChRs selectively stimulates effectors located near these AChRs that inhibit effectors located near glutamate receptors before they can be activated by calcium entering through these receptors. Evaluating therapeutic effects of any drug on AD, especially neuroprotective effects, is very difficult because extensive neurodegeneration may prevent acute beneficial effects and because long-term treatment of early diagnosed patients might be required to achieve benefit from any neuroprotective drug. At this point it is unclear whether AChR ligands will find any role in therapy of AD.

Parkinson's Disease

Parkinson's disease (PD) is a disease of extrapyramidal motor function characterized by difficulties in initiating and smoothly sustaining motions (Lloyd et al., 1975). It is associated with severe loss of dopamine-containing neurons in the substantia nigra. PD can be treated with the dopamine precursor L-DOPA, but this does not stop disease progression, its effectiveness ultimately decreases, and it may produce psychosis (Coleman, 1992). PD is also associated with a large (approx 50%) loss of high affinity nicotine binding sites from the brain (Whitehouse et al., 1988; Lang et al., 1993). Remarkably, tobacco use is the most potent environmental factor affecting susceptibility to PD, and is protective (Morens et al., 1995). This has led to some interest by SIBIA Neurosciences and other companies in developing AChR subtype-specific agonists as possible therapeutic agents for PD (Menzaghi et al., 1997a,b; Sacaan et al., 1997). The gastrointestinal and cardiovascular effects of nicotine limit its use for treating PD. The possible beneficial effects of a suitable agonist would include pre- and postsynaptic stimulation of dopamine release (Wonnacott, 1997) and possible neuroprotective effects (Akaike et al., 1994; Martin et al., 1994; Donnelly-Roberts et al., 1995). SIB-176F has high affinity for $\alpha4\beta2$ AChRs, low affinity for $\alpha7$ AChRs, and is at least as effective as nicotine in stimulating dopamine release from rat-brain striatal slices (Sacaan et al., 1997b). It also stimulates release of norepinepherine from cortex and thalamus and ACh from hippocampus and frontal cortex. SIB-176F has been reported to potentiate the effect of L-DOPA on the MPTP-induced model of PD in primates and on the reserpine-induced model in rats (Lloyd, 1996; Mezaghi et al., 1997). Quite which AChR subtype would be the ideal target for a therapeutic agonist in PD is unclear. For example, recent *in situ* hybridization studies have revealed the presence of $\alpha6$ in substantia nigra and some other regions that have previously been thought to contain $\alpha3$ using less specific *in situ* hybridization probes (LeNovere et al., 1996). Until quite recently $\alpha6$ was an orphan AChR subunit. Now it has been found able to function in combination with $\beta4$ subunits when expressed in *Xenopus* oocytes (Gerzanich et al., 1997). Although $\alpha6$ is closely related to $\alpha3$ in sequence, it differs

pharmacologically, which suggests the possibility that drugs specific for α6 AChRs could be developed. The native subunit composition and pharmacological properties of α6 AChRs are unknown. If they resembled those of α6β4 AChRs expressed in oocytes, and if such α6 AChRs were to play some role in PD, then these α6 AChRs might provide a useful pharmacological target with a distribution restricted largely to relevant regions of the brain and with distinct pharmacological properties that might allow design of specific ligands. It remains to be seen whether specific AChR ligands will find a place in the therapy of PD.

Schizophrenia

Schizophrenia is a devastating, heterogeneous psychosis beginning in late adolescence or early adulthood and running a chronic deteriorating course characterized by hallucinations, delusions, bizarre behavior, apathy, and blunted affect (Arnold and Trojanowski, 1996). A dopamine hypothesis of schizophrenia suggests that it is caused by excess dopamine. Some similar symptoms can also be caused by drugs like PCP that act as channel blockers for glutamate receptors and AChRs. Overall the pathological mechanisms are unclear. It has been suggested that α7 AChRs may be associated with some aspects of this disease (Leonard et al., 1996; Freedman et al., 1997). A high proportion of schizophrenics are intense tobacco users, and it has been suggested that they may be attempting to self medicate. It has been reported that there is a decrease in the normally high level of α7 AChRs in the hippocampus of brains of schizophrenia patients (Freedman et al., 1995). It has also been reported that a defect in auditory response that maps to the α7 gene locus may be a predisposing factor in schizophrenia (Freedman et al., 1997). The defect is a decrease in the normal inhibition of the P50 auditory-evoked response to the second of paired stimulae. It was suggested that this reflects attention disturbances in schizophrenia in which an inability to screen out irrelevant stimulae contributes to the development of hallucinations and delusions. In rats this response to paired stimulae involves the hippocampus, is blocked by αBgt, and inhibition of the response because of a fimbria-fornix lesion can be repaired by treatment with nicotine (Bickford and Wear, 1995). The proposal that α7 AChRs may be significantly involved in schizophrenia is a thought provoking proposal. The heterogeneity of this disease or group of diseases has long confounded attempts to associate it with specific biochemical lesions. The possible role of α7 AChRs in schizophrenia remains to be confirmed.

Tourette's Syndrome

Tourette's syndrome is a hyperkinetic motor disorder characterized by motor or verbal tics which start in childhood, as well as by the frequent co-occurrence of hyperactivity, anxieties, phobias, or obsessive-compulsive disorder. The pathological mechanisms are unclear. The dopamine receptor antagonist haloperidol can sometimes provide some benefit. Some open trial results report that nicotine patches alone or in combination with other pharmacological therapy can significantly reduce the incidence of tics in some patients (Silver et al., 1995; Sanberg et al., 1997). Although on acute exposure, nicotine is an agonist, on longer exposure it can cause reversible desensitization, and on prolonged exposure it can cause permanent inactivation of some AChR subtypes, especially α4β2 AChRs and α7 AChRs, but not α3 AChRs (Hsu et al., 1996; Olale et al., in press). Permanent inactivation of α4 or α7 AChRs coupled with a slow rate of resynthesis might account for the weeks of benefit reported for Tourette's syndrome patients treated for only 2 d with nicotine patches (Dursun et al., 1994). A controlled trial of nicotine in Tourette's syndrome has not been conducted, however these encouraging preliminary results suggest the possibility that nicotine or a more selective AChR ligand might prove useful in therapy of Tourette's syndrome.

Idiopathic Inflammatory Bowel Disease

The gut has as many neurons as the spinal cord and as many lymphocytes as the rest of the body (Furness and Costa, 1987). Many of the autonomic neurons in the gut may have AChRs, but this has not been well characterized. Smoking, perhaps via nicotine, has contrasting effects on two idiopathic inflammatory bowel diseases. On the down side, Crohn's disease occurs in higher incidence in smokers and may be exacerbated by smoking, whereas ulcerative colitis is largely a disease of nonsmokers, and smoking may even be protective against it (Thomas et al., 1995; Birtwistle, 1996). Treatment with transdermal nicotine patches, nicotine gum, and nicotine enemas are reported to be beneficial in these patients, but randomized double-blind studies have not been done (Thomas and Rhodes, 1995; Watson and Lewis, 1995; Guslandi and Tiltobello, 1996; Kennedy, 1996; Green et al., 1997). The mechanisms of nicotine's effects on ulcerative colitis are unknown.

Neuronal Nicotinic AChRs in Unexpected Locations

Neuronal AChRs may be found on cells not normally expected to contain AChRs (Sastry and Sadavongvidad, 1979), and this in some cases may prove relevant to disease. In the preceding discussion of MG, AChRs on lung small-cell carcinomas, other tumors, and in the thymus on myoid cells and thymocytes were mentioned as possible sources of immunogen for triggering the autoimmune response in this disease (Schluep et al., 1987; Luther et al., 1989; Taronni et al., 1992; Lennon et al., in press). Surprisingly, it has been reported that human foreskin keratinocytes secrete ACh and express α3, β2, β4, α5, α7, and α9 AChR subunits (Grando et al., 1995, 1996). ACh and nicotine increase calcium uptake by keratinocytes, alter gene expression, abolish migration, and increase adherence to other cells and substrate. Thus, ACh acting as a cytokine may be involved in keratinocyte differentiation and wound healing. Perhaps the high levels of butrylcholinesterase in blood and true ACh esterase expressed on erythrocytes reflects not only the need to remove any of this labile transmitter that might leak from neuronal sources despite their rich supply of ACh esterase, but also the need to destroy ACh involved in various cytokine effects to ensure that its effects remain local and specific.

Effects of Anesthetics

Volatile general anesthetics used to be thought of as acting through effects on cell membrane lipids, but membrane protein ion channels are increasingly thought to be the targets of action of this heterogeneous group of agents (Franks and Lieb, 1994). Investigations have focused primarily on their ability to potentiate and activate the function of inhibitory $GABA_A$ neurotransmitter receptors. However, recently it has been reported that α4β2 AChRs and, at lower sensitivity, α7 AChRs are potently inhibited by volatile anesthetics at concentrations lower than what are required for anesthesia (Flood et al., 1997; Violet et al., 1997). Anesthesia requires amnesia, analgesia, and sedation, and isoflurane suppresses memory at much lower concentrations than it inhibits responses to noxious stimulae (Evers and Steinbach, 1997). Thus, it has been suggested that AChRs might be relevant targets contributing to one of the components of anesthesia. In any case, experiments with chimeras nicely demonstrate that volatile anesthetics can exert their effects through the N-terminal extracellular domain of receptors (Zhang et al., 1997). α7 AChRs, $5HT_3$ serotonin receptors, and $GABA_A$ receptors all belong to the same gene superfamily of ligand-gated neurotransmitter receptors (Lindstrom, 1996). The homologies in their structures were elegantly illustrated by the demonstration that a chimera between the N-terminal extracellular half of α7 subunits and the C-terminal half of $5HT_3$

receptors containing the region M1 through the C-terminus can form functional homomeric ACh-gated channels with the conductance properties of $5HT_3$ receptors (Eisile et al., 1993) as well as by the demonstration that substituting three amino acids from the M2 domain of the anion-selective receptors for GABA and glycine into the M2 domain of the cation selective channels of α7 converts the mutant α7 AChRs to anion-selective channels (Galzi et al., 1992). Volatile anesthetics inhibit α7 AChRs, but potentiate $5HT_3$ receptors. In the case of the chimera, volatile anesthetics inhibited, showing that they acted on the N-terminal extracellular part of the chimera derived from α7 AChRs (Zhang et al., 1997).

Effects of Ethanol

Ethanol is a drug at least as diffuse in its actions and as impotent as volatile anesthetics. Ethanol requires relatively huge concentrations (1–10 m*M*) to produce many of its common effects (Little, 1991). The sites of its actions are unclear. Ethanol and nicotine are the two most abundant abused drugs. Ethanol use increases voluntary smoking (Heningfield et al., 1984), nicotine increases voluntary ethanol intake in rats (Pottholf et al., 1983), and there is cross tolerance between ethanol and nicotine in mice (Collins et al., 1987). Both drugs activate the mesolimbocortical dopamine system associated with the reinforcing properties of abused drugs like cocaine (Blomquist et al., 1993; Pich et al., 1997). Mesolimbic dopamine activation by ethanol can be prevented by the AChR-channel blocker mecamylamine (Blomquist et al., 1993). Very low concentrations of ethanol (EC_{50} = 89 μ*M*) were reported to inhibit the α3 AChRs of rat PC12 cells by increasing the rate of desensitization (Nagata et al., 1996). Thus, it was suggested that α3 AChRs may be involved in the early euphoric effects of ethanol. Ethanol, like volatile anesthetics, apparently acts on the extracellular domain of AChRs (Zhang et al., 1996). This was similarly shown using α7 AChR/$5HT_3$R chimeras. Ethanol noncompetitively inhibits α7 AChRs with an EC_{50} of 33 m*M*, but potentiates $5HT_3$R responses with an EC_{50} of 57 m*M*. A chimera with the N-terminal extracellular domain of α7 and M1 through the C-terminus of the $5HT_3$R is inhibited by ethanol as efficiently as are intact α7 AChRs.

Effects of Nicotine

By far the largest medical effect involving AChRs is the mediation of addiction to tobacco (Koop et al., 1988), which has been reported to account for more than 400,000 premature deaths per year in the United States and 250,000,000 premature deaths worldwide by the turn of the century (Peto et al., 1992). Nicotine affects most organ systems in the body, but its direct contributions to tobacco-related disease processes are unclear (Benowitz, 1996). It does increase heart rate and blood pressure (Benowitz, 1996). There are a few reports of nicotine actually behaving as a mitogenic factor under certain circumstances in some lung carcinomas (Codingnola et al., 1994; Schuller, 1995).

Nicotine as a drug may have received a "bum rap" through its mediation of addiction to tobacco. Nicotine as a medication is primarily used as part of smoking cessation therapy in the form of gum, transdermal patches, or most recently, a nasal spray (Benowitz, 1996). As discussed in previous sections, nicotine has been reported to show some benefit in therapy of Alzheimer's disease, Parkinson's disease, Tourette's syndrome, ulcerative colitis, sleep apnea, and attention deficit disorder (Benowitz, 1996). In these cases nicotine may be most valuable as a lead compound to suggest that development of more subtype-specific AChR agonists or antagonists will be more therapeutically useful through more potent beneficial effects and diminished side effects. The discovery of many AChR subtypes, some of which are associated with only a few brain nuclei, suggests that it may be possible to develop very specific drugs. The presence of AChRs in many tissues suggests that it may be possible to influence many different processes

through AChR ligands. However, one of the major problems is that the precise physiological roles of AChRs in brain and several other tissues remain unclear. Nonetheless, the huge public health impact of nicotine's role in tobacco addiction demonstrates that the physiological roles of AChRs in brain can be very important.

The mesolimbic dopamine system is thought to mediate dependence on both nicotine and other drugs of abuse (Pich et al., 1997). In smokers, nicotine use is thought to be motivated both by positive and negative reinforcement (Benowitz, 1996). Positive reinforcing factors are relaxation, reduced stress, increased vigilance, improved cognition, and reduced body weight. The potent effects of nicotine on reducing body weight through increased metabolic rate and decreased consumption of sweets, which are thought to be especially important in encouraging young females to smoke, also suggest a possible use of an appropriate subtype-specific AChR drug (Williamson et al., 1991; Perkins, 1992; Aracavi et al., 1993). Negative reinforcing factors promoting tobacco use are relief from unpleasant withdrawal symptoms including nervousness, restlessness, irritability, anxiety, impaired concentration and cognition, and weight gain (Hughs and Hatsukami, 1986).

Determining the mechanisms by which chronic use of nicotine produces both reward and tolerance is complicated by three major factors: the existence of multiple subtypes of AChRs that differ in their responses to nicotine (Sargent, 1993; McGehee and Role, 1995; Lindstrom, 1996); the ability of nicotine and other agonists to activate AChRs on acute exposure, to reversibly desensitize them on longer exposure, and to permanently inactivate them after prolonged exposure (Lukas, 1991; Collins and Marks, 1996; Dani and Heinemann, 1996; Hsu et al., 1996); and the ability of prolonged exposure to nicotine and other agonists to cause an increase in the amount of AChRs (Flores et al., 1992; Marks et al., 1992; Peng et al., 1996, 1997). This raises the basic question of which effects of nicotine are caused by a net activation of AChRs and which are caused by a net inactivation of AChRs (Collins and Marks, 1996; Dani and Heinemann, 1996). Rewarding effects leading to self administration of nicotine are reported to be blocked by pretreatment with the ACh-channel blocker mecamylamine (Henningfield, 1984), suggesting that activation of some AChR subtype is important in some aspects of reinforcement. Reversible desensitization of AChRs by exposure to nicotine, and especially net permanent inactivation of AChRs despite upregulation in amount resulting from prolonged exposure to nicotine, offer obvious explanations for the development of tolerance to nicotine in chronic users who become insensitive to nausea and other aversive effects of nicotine on naive users (Hsu et al., 1996; Olale et al., in press). Recovery of function of reversibly desensitized AChRs after overnight abstinence might explain the reported perception of the first cigarette of the day as the best one either because of recovery of AChR subtypes available for activation to mediate rewarding effects of nicotine or recovery of AChR subtypes available for desensitization that mediate aversive withdrawal effects of nicotine, or by a mixture of both types of processes.

The unique dosage pattern with nicotine provides further complications in understanding its effects (Benowitz, 1990, 1996). Nicotine is a tertiary amine that can rapidly cross cell membranes. Consequently, within a minute of inhaling, smokers can bathe their brains in μM nicotine. Liver metabolism rapidly converts nicotine to cotinine, but smokers can maintain a sustained serum concentration of 0.2 μM nicotine. Smoking patterns differ between individuals, suggesting that different folks seem to strike different chords among the many possible notes provided by the effects of different AChR subtypes. For example, the sustained serum levels of nicotine could maintain tolerance and some positive reinforcements through net inactivation of some AChR subtypes through desensitization and inactivation, whereas the high-dose boluses of nicotine experienced during inhaling could mediate other positive reinforcements through activation of AChR subtypes

that were less subject to desensitization or inactivation and perhaps also more sensitive to upregulation in amount.

It is instructive to compare properties of the three most prominent neuronal AChR subtypes: α4β2 AChRs, α7 AChRs, and α3 AChRs (*see* Fig. 1). α4β2 AChRs represent >90% of the high affinity nicotine binding sites in mammalian brain (Whiting and Lindstrom, 1988). α7 AChRs represent >90% of the high affinity αBgt binding sites in brain, are present in brain in about equal amounts with α4β2 AChRs in a similarly wide ranging and overlapping but distinct distribution, and are often found in peripheral autonomic neurons along with α3 AChRs (Clarke et al., 1985; Schoepfer et al., 1990; Conroy and Berg, 1995). α3 AChRs are present in brain in smaller amounts and more limited distribution than are the other two subtypes, but they are probably the predominant postsynaptic AChR in autonomic neurons (Sargent, 1993). Nicotine has the highest affinity and potency on α4β2 AChRs, but lower affinity and potency on the other two subtypes. Nicotine on cloned human α4β2 AChRs has an EC_{50} for activation of 2.2 μ*M* (Olale et al., in press) and a KI for binding of 0.001 μ*M* (Gopalakrishnan et al., 1996). Nicotine on cloned human α7 AChRs has an EC_{50} for activation of 40 μ*M* and a KI for binding of 1.6 μ*M* (Peng et al., 1994b; Gopalakrishnan et al., 1995). Nicotine on cloned human α3β2 AChRs has an EC_{50} for activation of 6.8 μ*M* and an efficacy of 50%. On α3β2α5 AChRs nicotine has an EC_{50} of 1.9 μ*M* and is fully active (Wang et al., 1996). On human α3β4 AChRs nicotine has an EC_{50} for activation of 106 m*M*, and on α3β4α5 AChRs nicotine has the same potency and is 100% effective on both subtypes (Wang et al., 1996). A typical neuronal cell line like the human neuroblastoma SH-SY5Y contains a mixture of α3β2, α3β2α5, α3β4, and α3β4α5 AChRs in which β2 containing AChRs comprise 56% of the total α3 AChRs (Wang et al., 1996). The KD for binding of nicotine to α3 AChRs solubilized from SH-SY5Y is 0.02 μ*M* (Peng et al., 1997). Chronic nicotine exposure increases the amount of brain high affinity nicotine binding sites (i.e., primarily α4β2 AChRs) by up to two fold while producing a smaller increase in the amount of αBgt-binding sites (i.e., α7 AChRs) (Flores et a., 1992; Marks et al., 1992). This is not because of an increase in transcription of α4 and β2 subunits (Marks et al., 1992). Instead, this results from a decrease in the rate of turnover of α4β2 AChRs on the cell surface (Peng et al., 1994a). This effect is thought to result from a nicotine-induced conformation change, perhaps to a desensitized or inactivated conformation, rather than to result from signals mediated by ion flow through the α4β2 AChR channel, because the channel blocker mecamylamine can also induce upregulation of both cloned α4β2 AChRs and brain AChRs (Peng et al., 1994a; Pauly et al., 1996). The EC_{50} for nicotine causing a twofold upregulation of cloned α4β2 AChRs over 2 d is 0.2 μ*M* (Peng et al., 1994a), a relevant concentration equal to that found in a typical smoker's serum (Benowitz et al., 1990). Chronic nicotine treatment also upregulates the α3 AChRs and α7 AChRs found in the human neuroblastoma cell line SH-SY5Y (Peng et al., 1997). This requires higher nicotine concentrations than are typical of smoker's serum (i.e., EC_{50} for α3 AChR upregulation = 100 μ*M* and EC_{50} for α7 AChR upregulation = 65 μ*M*). This suggests that in brains of humans exposed to nicotine there might be little upregulation of α3 and α7 AChRs. The extent of nicotine-induced upregulation of α3 AChRs solubilized from SH-SY5Y cells was very large (500–600%), whereas the extent of upregulation of α7 AChRs was quite small (30%) (Peng et al., 1997). In this cell line, it was found that the increase of α3 AChRs induced by nicotine involved α3 AChRs that appeared transiently on the cell surface, then accumulated inside the cell. Their exposure on the cell surface was shown by their susceptibility to antigenic modulation by a MAb to the MIR. The mechanisms of upregulation of α3 AChRs and α7 AChRs appear to differ from those of α4β2 AChRs not only in nicotine sensitivity, extent, and surface expression, but also pharmacologically in that mecamylamine did not induce upregulation.

Chronic exposure to nicotine differentially affects the desensitization and inactivation of human α4β2 AChRs, α7 AChRs, and α3 AChRs (Hsu et al., 1996; Olale et al., in press). After 3 h or 24 h in the 0.2 m*M* concentration of nicotine typical of smoker's serum, approx 80% of the current inducable by a saturating concentration of ACh on α4β2 AChRs or α7 AChRs was lost and not recoverable even after 24 h of washing (Olale et al., in press). However, α3 AChRs were inhibited only slightly by 3 h in this concentration of nicotine and recovered completely after 1 h of washing (Olale et al., in press). The α3β2 and α3β2α5 AChR subtypes (that have higher affinity for nicotine) were inhibited approx 55% in the presence of 0.2 μ*M* nicotine for 3 h, whereas the α3β4 and α3β4α5 AChR subtypes (that have lower affinity for nicotine) were not inhibited at all by this low nicotine concentration (Olale et al., in press).

These results suggest that in a typical smoker with a 0.2 μ*M* serum concentration of nicotine, virtually all of their α4β2 AChRs and α7 AChRs would be inactivated, whereas most of their α3 AChR subtypes would be available to respond to endogenous ACh or boluses of nicotine either immediately or after a short recovery period. Development of tolerance may thus depend on inactivation of α4β2 AChRs and/or α7 AChRs. It is reasonable to suppose that net loss of these AChR subtypes could occur without major neurological impairment because in mice neither knockout of the β2 subunit gene that eliminates the high affinity nicotine binding expected of α4β2 AChRs (Picciotto et al., 1995) nor knockout of the α7 subunit gene (Orr-Urtriger et al., 1996) causes gross behavioral or brain anatomical anomalies.

It is evident that the effects of nicotine on AChRs are complex at the molecular level, making it more difficult to sort out the effects of nicotine at the synapse, cell, or system levels. As an example of the complexities in looking even at the synapse level, consider the complexities resulting from the expression of multiple AChR subtypes within a single neuron. At ciliary ganglia synapses either postsynaptic α3 AChRs or perisynaptic α7 AChRs can successfully mediate transmission (Zhang et al., 1996). In such a synapse chronically exposed to nicotine the safety factor in transmission provided by α7 AChRs would be lost, but transmission mediated by α3 AChRs would continue.

Any subtype-specific AChR agonist that might be developed for use as a drug would be expected to encounter issues of acute activation vs chronic inactivation, upregulation, excitotoxicity, and so on, similar to those encountered with nicotine. However, specificity for a single AChR subtype would result in a more nearly monotonic effect. The adverse medical consequences of AChR involvement in smoking could be eliminated by eliminating smoking. More effective use of nicotinic ligands and means of their delivery may help in smoking cessation. For example, to deal with the complexities of nicotine's effects, combined therapy with the agonist nicotine and the antagonist mecamylamine has been investigated as a more effective approach to smoking cessation (Rose et al., 1995).

The potentially beneficial consequences of subtype-specific nicotinic drugs are hinted at by some of the many effects of nicotine.

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