CHAPTER

13

Acetylcholine

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OUTLINE

INTRODUCTION

Acetylcholine was first synthesized by Adolf von Baeyer in 1867, long before a biological role for this molecule had been established. In 1914, ACh was discovered in ergot-containing plant material and Dale noted that when applied to peripheral tissues, ACh had the same effect as that of the stimulation of parasympathetic nerves (Dale, 1914). However, it was Loewi, in 1921, who unequivocally demonstrated that nerve-to-muscle signaling in the heart was mediated by a chemical substance, originally termed 'vagusstoff' and subsequently determined to be ACh. In these seminal experiments, Loewi was able to demonstrate that when the parasympathetic nerve to the heart (vagus nerve) was stimulated, a substance was released into the surrounding fluid that, when applied to a denervated second heart, resulted in its slowing (Loewi, 1921). Although these experiments were the first to demonstrate neurohumoral transmission, it was not until 1929 that ACh was first isolated from animal tissues (Dale & Dudley, 1929). In 1936, Dale and colleagues chemically identified ACh as the neurotransmitter at the neuromuscular junction (NMJ; Dale et al., 1936). For their work on ACh, both Loewi and Dale received the Nobel Prize in Physiology or Medicine. It is now known that in addition to its widespread role as a transmitter within the CNS and at the NMJ, ACh also serves as a neurotransmitter at peripheral ganglia and as a mediator of the parasympathetic actions of the autonomic nervous system. It is likely that ACh was an early organic molecule to evolve since, in addition to its established presence within the nervous system, it is also synthesized in bacteria, fungi, protozoa and plants. Perhaps as a result of ACh's early appearance during evolution, a number of toxins that interfere with the action of ACh at the NMJ that are used to advantage by predators have been identified. ACh is also found in non-neural tissues such as epithelial cells, cornea and placenta, although the physiological significance remains unclear.

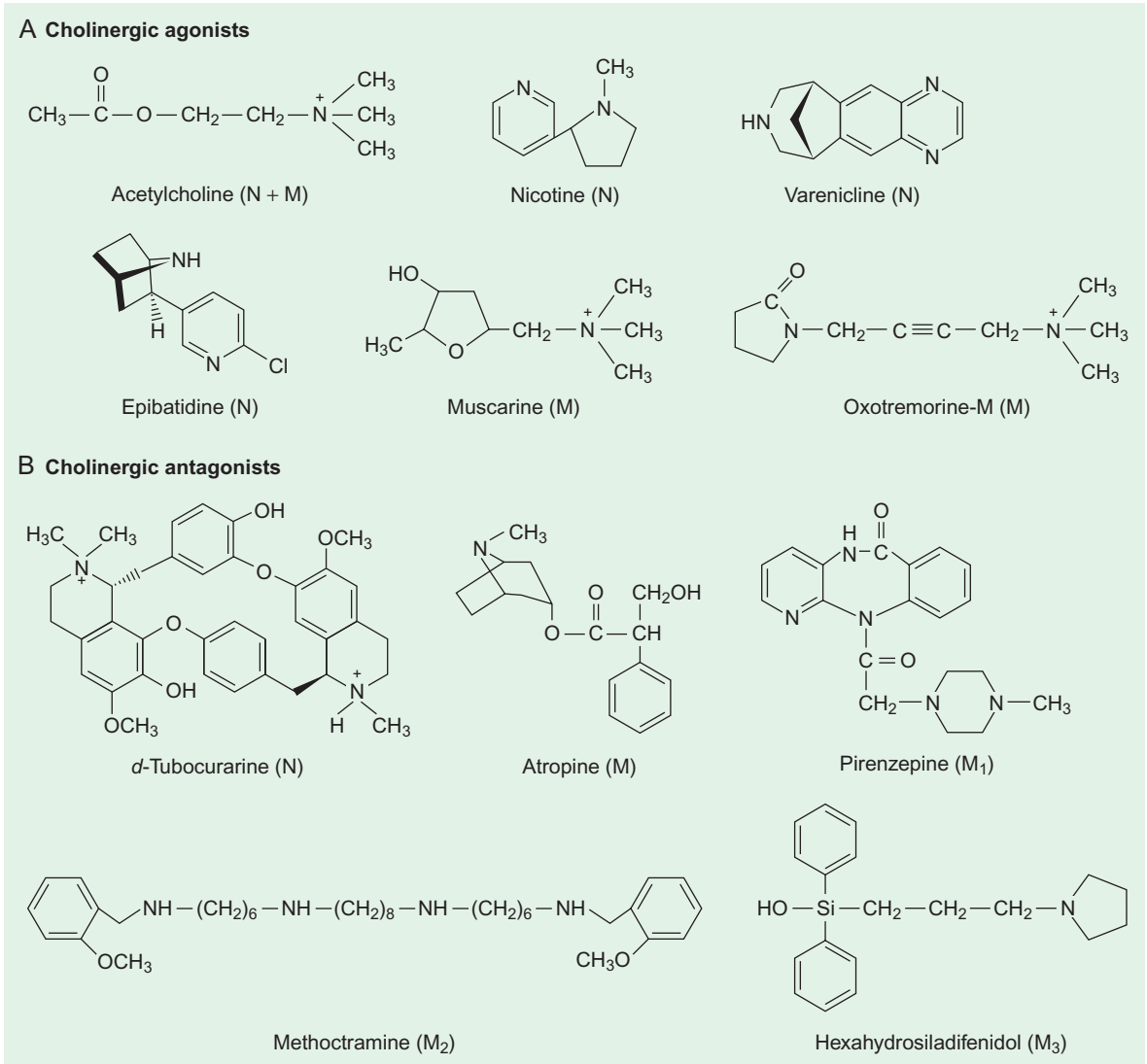


FIGURE 13-1 **Structures of cholinergic agonists and antagonists.** Compounds are subdivided into nicotinic (N) or muscarinic (M) categories or both, as in the case of ACh. Compounds with muscarinic receptor selectivity (M1, M2, M3, M4) are also noted.

In addition to its pivotal role in the study of chemical transmission, ACh was instrumental in the pharmacological classification of receptors. In 1905, Langley demonstrated that the direct application of nicotine caused skeletal muscle to contract, an observation that led him to postulate the presence of a 'receptive substance' in the muscle membrane (for review, see Maehle et al., 2002). Subsequent studies by Langley (1906) and Dale (1914) indicated that whereas some effects of ACh were mimicked by the addition of nicotine, others were replicated by the alkaloid muscarine (see Fig. 13-1 for chemical structures). These observations led to the concept that different subtypes of cholinergic receptors, namely nicotinic (nAChR) and muscarinic (mAChR), were present in tissues. As detailed below, both nAChRs and mAChRs have been extensively studied and information gathered on these receptor subtypes has been used to advantage in enhancing our understanding of those pathological states that involve the cholinergic system, as well as the identification of therapeutically beneficial agents.

SYNTHESIS, STORAGE AND RELEASE OF ACETYLCHOLINE: DISTRIBUTION OF CHOLINERGIC PATHWAYS

Acetylcholine formation is catalyzed by choline acetyltransferase

The enzyme choline acetyltransferase (ChAT; EC 2.3.1.6) catalyzes the reaction acetyl-CoA + choline → acetylcholine. Although this reaction is reversible, the equilibrium is strongly shifted in the direction of ACh formation. Brain ChAT is a single subunit globular protein with MWs ranging from 67–75, depending on the species. The enzyme has been purified and cloned and this has allowed for the development of highly specific antibodies that have greatly facilitated the mapping of cholinergic pathways within the CNS. ChAT is synthesized within the rough endoplasmic reticulum in the cell body and then transported by axoplasmic transport (see Chap. 8) to the nerve terminal, where it becomes highly concentrated within the cytoplasm. When brain is homogenized in an isotonic medium and the homogenate subjected to differential and density gradient centrifugation, a large proportion of the ChAT activity is recovered in a synaptosomal (pinched-off nerve terminals) fraction. Brain regions that possess a high ChAT activity may either be the site of termination of cholinergic projection neurons where ACh is released (e.g., hippocampus) or, alternatively, reflect the presence of a significant number of cholinergic interneurons within the structure (e.g., caudate/putamen). In general, measurement of the activity of ChAT, an enzyme restricted to presynaptic structures, in a given brain region is a good predictor of the likelihood of cholinergic innervation. In contrast, the activity of acetylcholinesterase (AChE), the degradative enzyme, is not a reliable indicator of cholinergic innervation since the enzyme is found at both pre- and postsynaptic locations. For example, AChE is highly enriched in the substantia nigra even though this structure is only sparsely innervated by cholinergic neurons.

The two substrates utilized by ChAT, namely acetyl-CoA and choline, are derived from intra- and extracellular sources, respectively. Acetyl-CoA is synthesized predominantly via the pyruvate dehydrogenase complex located in the intra-terminal mitochondria. From there, acetyl-CoA is translocated into the cytoplasm via an unknown mechanism. In contrast, the source of choline is extra-synaptosomal. Although non-neural tissues can synthesize choline from successive methylation of ethanolamine, this pathway does not make a significant contribution to choline content in the brain. Furthermore choline is not readily taken up across the blood–brain barrier. Rather, it appears that the major source of choline for ACh synthesis is that derived from AChE-mediated hydrolysis of ACh, following the release of the transmitter into the synaptic cleft. As detailed below, the uptake of choline into the nerve terminal is mediated by a specific transporter. The K_m values of acetyl-CoA and choline for ChAT activity are 0.4–1 mM and 7–46 μM, respectively, whereas the cytoplasmic concentrations of these substrates are 50 μM and 5 μM, respectively. Thus, ChAT activity is likely to be regulated by substrate availability.

Choline is accumulated into synaptic terminals via a specific high-affinity transporter

The uptake of choline can occur via two transport systems, one that exhibits a high affinity for choline uptake (HAChU, K_m = 1–5 μM) and is dependent on both Na^+ and Cl^-, and a second transporter system that has a significantly lower affinity for choline uptake (LAChU, K_m = 10–100 μM) and is independent of Na^+ and Cl^- (see also Ch. 3). LAChU is found in all tissues and is primarily linked to the synthesis of phosphatidylcholine, whereas HAChU is restricted to cholinergic nerve terminals. A potent inhibitor of HAChU (but not of LAChU) is hemicholinium-3, and this agent has been used to deplete tissues of their ACh content.

HAChU is mediated by a high-affinity choline transporter (ChT) that was first cloned by Okuda and colleagues (Okuda et al., 2000). ChT, which has a MW of 63, is a member of the Na^+/glucose solute carrier superfamily (SLC5, Ch. 3). Analysis of the amino acid sequences predicts a topology of 13 transmembrane domains, an extracellular glycosylation site, cytoplasmic phosphorylation sites for protein kinases A and C and a di-leucine–like internalization motif in the cytoplasmic C-terminus region. The concentration of choline in the plasma is approximately 10 μM, a concentration in excess of the K_m for choline uptake. Thus, under normal conditions, ChT would be expected to be saturated with choline and operating maximally. However, HAChU demonstrates considerable plasticity and can be increased by both behaviorally and pharmacologically induced activation of cholinergic neurons (Murrin & Kuhar, 1976; Apparsundaram et al., 2005). In contrast, depression of cholinergic neuronal activity results in suppression of both choline transport and ACh synthesis. Two explanations have been proposed to account for the increase in HAChU with activation of cholinergic neurons, one being an increase in the catalytic activity of ChT (post-translational modification), and the other being an altered trafficking of ChT with an accumulation of the transporter at the plasma membrane. The latter explanation appears to be the case, since the development of ChT-specific antibodies has revealed that the ChT is localized to two distinct pools: one intracellular pool associated with the cholinergic

vesicles (that constitutes the majority of the transporter) and one at the plasma membrane. Experiments with transfected cell lines and primary neuronal cultures indicate that ChT is internalized by means of clathrin-mediated endocytosis. Stimuli that facilitate ACh release result in the appearance of ChT at the plasma membrane (Fig. 13-2). Thus HAChU is tightly coupled to ACh release and is the rate-limiting factor in ACh synthesis. The importance of ChT is evident from the fact that genetic ablation of ChT is lethal and mice die within an hour of birth, due to their inability to sustain ACh synthesis and release at the NMJ. This leads to motor and respiratory paralysis (Bazalakova & Blakely, 2006).

ACh is packaged into vesicles by a specific transporter and is released from neurons in a Ca^{2+}-dependent manner

A specific vesicular ACh transporter (VAChT) mediates the transfer of ACh from the cytoplasm into synaptic vesicles. VAChT is an antiporter that couples ACh entry into the vesicle with proton efflux, the same mechanism used for the accumulation of other biogenic amine neurotransmitters (see Chap. 3). This mechanism allows for the accumulation of ACh at high concentrations, which in the electric ray *Torpedo* (see Fig. 13-8) can reach 1 M. Vesamicol is an agent that selectively inhibits the vesicular ACh transporter. The effectiveness of this drug to block ACh storage can be observed at the NMJ. Under normal conditions, rapid stimulation of the cholinergic neuron does not affect the amplitude of the subsequent miniature end plate potentials (MEPPs: see Chap. 12). In contrast, when vesamicol is present, the size of the subsequent MEPPs is substantially reduced due to the inhibition of vesicle refilling and a decrease in quantal size. From cloning studies, it can be deduced that VAChT possesses 12 putative transmembrane spanning domains, a characteristic shared with other biogenic amine transporters. The gene coding for VAChT is located within an intron of the ChAT gene, which may indicate that ChAT and VAChT are coregulated. The synaptic vesicles in which ACh is packaged also possess a number of intrinsic proteins involved in the release of the neurotransmitter upon nerve stimulation. These include the synaptobrevins, synaptophysins and synaptotagmins (see Chap. 7).

ACh is released in packets or 'quanta' from the nerve terminal (Ch. 12). Cholinergic vesicles, identified from biochemical and electron microscopic approaches, constitute the structural basis for these discrete units of ACh. Mammalian cholinergic vesicles contain approximately 2,000 molecules of ACh, whereas those obtained from *Torpedo* contain approximately 20,000 molecules.

The availability of extracellular Ca^{2+} is a major determinant in the release of ACh. Upon depolarization of a neuron, Ca^{2+} enters the presynaptic terminal and facilitates the fusion of the vesicle membrane with the plasma membrane. ACh is then released into the synaptic cleft via exocytosis (see Chap. 7). This process is inhibited by botulinum neurotoxins (BoNTs; types A–G) produced by the anaerobic bacterium, *Clostridium botulinum*, which are toxic because they inhibit ACh release from peripheral cholinergic neurons, notably motor neurons

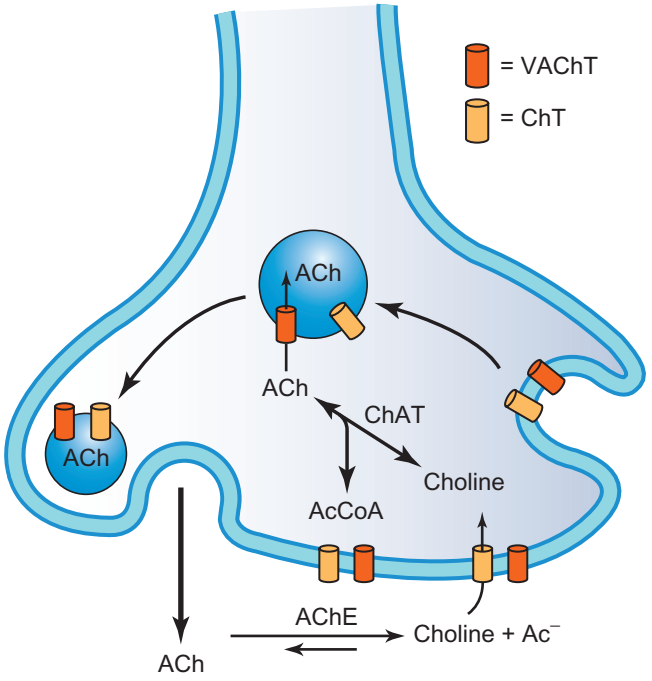


FIGURE 13-2 Transport, synthesis and degradative processes in a cholinergic presynaptic nerve terminal and synapse. The choline transport protein (ChT) functions at the nerve ending membrane to transport choline into the cytoplasm, where its acetylation by acetyl CoA is catalyzed by choline acetyltransferase (ChAT) to generate acetylcholine (ACh) in the vicinity of the synaptic vesicle. The vesicular acetylcholine transporter (VAChT) concentrates acetylcholine in the vesicle. ChT is also found on the vesicle but in a functionally inactive state. Upon nerve stimulation, depolarization and Ca^{2+} entry, ACh-containing vesicles fuse with the membrane and release their contents. The fusion of the membrane results in more ChT being exposed to the synaptic gap, where it becomes active. ACh is hydrolyzed to acetate and choline catalyzed by acetylcholinesterase (AChE), allowing for recapture of much of the choline by ChT. Because of the differing ionic compositions in the extracellular milieu and within the cell, ChT is thought to be active only when situated on the nerve cell membrane. Similarly, the VAChT may only be active when encapsulated in the synaptic vesicle (Ferguson & Blakely, 2004).

(Verderio et al., 2006; see Chap. 44 for mechanism). BoNTs are extremely potent (LD_{50} values for mice range from 0.5 to 5.0 ng/Kg) and can lead to muscular paralysis and death by asphyxiation. Although BoNTs can also inhibit neurotransmitter release from noncholinergic nerve terminals, their toxicological effects are chiefly observed at peripheral synapses where cholinergic innervation predominates. Despite the fact that BoNTs are some of the most potent toxins known, their application (albeit at very low concentrations) can also be of clinical efficacy in the treatment of a large number of conditions due to the overactivity of nerves supplying various muscles and glands. In the United States, Botox is currently approved for five defined uses (frown lines, crossed eyes, eyelid twitching, neck muscle contractions and severe underarm sweating), but many other uses have been approved in other countries. Tetanus toxin, which like BoNT acts as a Zn^{2+} endopeptidase, also inhibits the release of ACh (and other neurotransmitters) by targeting synaptobrevin, although its principal targets in

cases of tetanus poisoning are the inhibitory synapses of the spinal cord. This results in a rigid paralysis in contrast to the flaccid paralysis produced by BoNT.

Not all ACh within the nerve terminal is available for release, and the presence of 'depot' and 'reserve 'pools of ACh has been proposed. However, the relationship between these putative pools of ACh and populations of cholinergic vesicles has yet to be defined.

Cholinergic neurons are widely distributed within the CNS

Although the identification of cholinergic pathways terminating in the peripheral nervous system, e.g., spinal and cranial motor neurons, has proven to be relatively straightforward, the mapping of cholinergic neurons within the CNS has been more difficult. The traditional approach has involved creating a lesion in a putative cholinergic tract, and then taking biochemical measurements of ACh content or ChAT or HAChU activities in the presumed projection area, with a reduction in any of these parameters in the target area indicating that cholinergic neurons terminated at that site. The availability of highly specific antibodies to ChAT has permitted the emergence of a more definitive picture of cholinergic tracts within the CNS. The major cholinergic neuronal pathways in the CNS are shown in Fig. 13-3. Projection neurons of the basal forebrain include (1) cholinergic cell groups located in the medial septal nucleus and the vertical limb of the diagonal band of Broca, which send axons to the hippocampus and limbic cortex; (2) cholinergic neurons found in the horizontal nucleus of the diagonal band and the magnocellular preoptic area, whose axons terminate in the olfactory bulb, amygdala and limbic cortex; and (3) cholinergic cell bodies in the nucleus basalis of Meynert, which project to all parts of the neocortex. As detailed below, the latter pathway is of particular significance in Alzheimer's disease (see also Chap. 46). A second group of cholinergic projection neurons originate in the pedunculopontine tegmental nucleus and the dorsolateral tegmental nucleus. These cell bodies project to many areas of the CNS, chief of which is the thalamus. These cholinergic pathways are of particular relevance to cortical arousal and sleep (see Chap. 57). In addition to these two groups of cholinergic projection neurons, a third group of cholinergic neurons is located in the striatum. However, these are interneurons, rather than projection neurons, and are involved in the local circuitry. These cholinergic neurons are of importance in the functioning of the extrapyramidal motor system and can become overactive in Parkinson's disease (see Chap. 49).

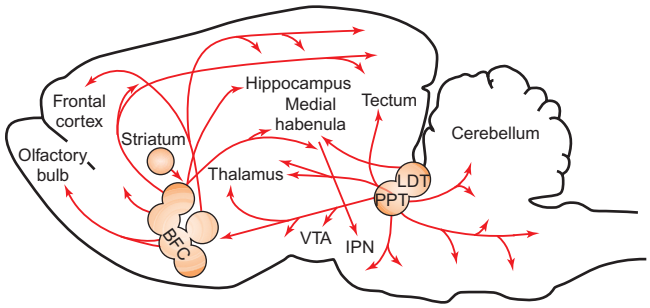


FIGURE 13-3 Major cholinergic pathways in the rat brain. The principal source of cholinergic input to the cerebral cortex and hippocampus is the basal forebrain complex (*BFC*). Cell bodies in the nucleus basalis of Meynert project to the neocortex whereas cell bodies in the horizontal nucleus of the diagonal band and the magnocellular preoptic area project to the olfactory bulb, amygdala and limbic cortex. Cholinergic cell bodies located in the medial septal nucleus and vertical limb of the diagonal band project to the hippocampus and limbic cortex. The pedunculopontine and laterodorsal tegmental areas (PPT and LDT, respectively) preferentially innervate the brain stem and midbrain targets. Cholinergic interneurons predominate in the striatum. VTA, ventral tegmental area, IPN, interpeduncular nucleus. (Adapted with permission from the American College of Neuropsychopharmacology (ACNP), from Neuropsychopharmacology: The Fifth Generation of Progress, Picciotto et al., 2002).

ENZYMATIC BREAKDOWN OF ACETYLCHOLINE

Acetylcholinesterase and the removal of ACh

ACh is eliminated from CNS and peripheral synapses through enzymatic hydrolysis by acetylcholinesterase (AChE; EC 3.1.1.7).

$$ACh + H_2O \rightarrow Choline + Acetate$$

This is unusual because all other nonpeptide neurotransmitters are primarily removed by transporter-mediated reuptake into surrounding nerve terminals or glial cells. The enzymatic degradation of ACh may have evolved to satisfy the need for the very rapid removal of ACh at the neuromuscular junction, within a few milliseconds of release, in order to achieve exquisite temporal regulation of muscle contraction. To this end, AChE is a highly efficient enzyme that exhibits near perfect catalytic activity, reflected in the ratio of k_{cat}/K_m of $10^8 M^{-1}s^{-1}$, where k_{cat} is the turnover number and K_m is the affinity for ACh. This value approaches the diffusion-controlled limit set by the rate of diffusion of substrate into the active site (Taylor & Radic, 1994). The desire to understand how such perfection is achieved, together with the drive to target AChE for clinical benefit or toxicological purposes, have stimulated research into the structure and function of this enzyme.

Molecular forms of AChE

AChE is found in many different locations throughout the body: in addition to the NMJ and the central and peripheral nervous systems where cholinergic transmission occurs, it is also associated with non-neuronal tissues including red blood cells and placenta. Biochemical experiments have revealed that AChE exists in a variety of molecular forms, illustrated in Fig. 13-4 (Massoulié, 2002). The 'catalytic subunit' that is common to all forms can exist as monomers, dimers or tetramers (the multimeric forms are held together by covalent disulfide bonds). The dimeric form can differ in whether or not it has the post-translational attachment of glycophospholipid to the carboxyl terminus. This glycophosphatidylinositol (GPI; see

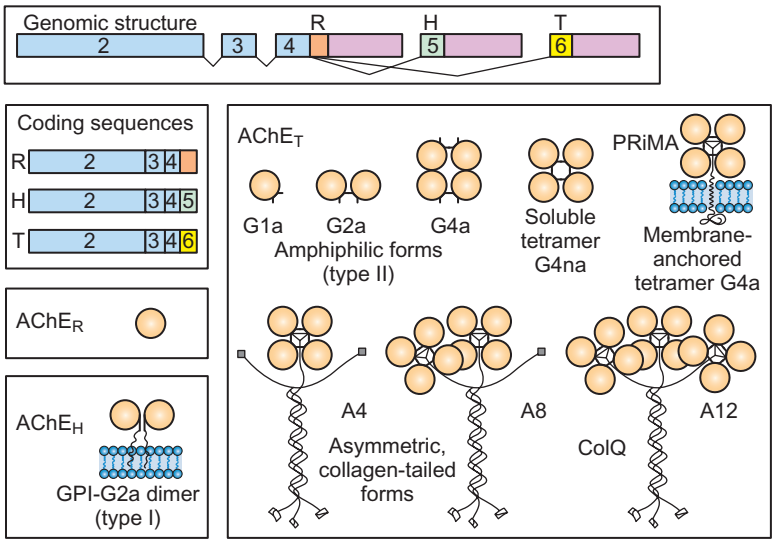


FIGURE 13-4 Molecular forms of AChE. The genomic structure (top) shows three exons, 2,3 and 4, which encode the invariant catalytic domain, followed by three splicing alternatives. (Exon 1, not shown, encodes the N-terminal signal peptide and does not contribute to the mature enzyme.) The resultant forms of AChE are a soluble, monomeric form that terminates after exon 4 (R, 'readthrough'); a 'hydrophobic' form (H) that includes exon 5, which permits dimerization and attachment of a GPI anchor; and the most prevalent form (T), which contains exon 6. This exon codes for a 40-amino acid T peptide that allows oligomerization of globular (G) forms and attachment of tetramers to hydrophobic tails (*either ColQ or PRiMA*) to generate asymmetric (A) forms. Adapted from Massoulié, 2002.

Chap. 5) anchor intercalates into the membrane, allowing the enzyme to be tethered to the external surface of the plasma membrane. This form of AChE is found on red blood cells.

The catalytic subunit also forms heteromeric assemblies with structural proteins that can tether AChE extracellularly in different ways. These versions of the enzyme are sometimes termed 'asymmetric' because the structural tail protein confers substantial 3-dimensional asymmetry. The predominant form at the neuromuscular junction consists of 1, 2 or 3 tetramers of catalytic subunits associated with a collagen tail, known as ColQ, through disulfide bonding and a coiled-coil mechanism. In an AChE tetramer of catalytic subunits, the four C-terminal domains (each consisting of 40 amino acids in an α-helical formation and termed the T-peptide) form a cylinder around a **p**roline-**r**ich N-terminal **a**ttachment **d**omain (PRAD) of ColQ (Massoulié & Millard, 2009). Three ColQ strands entwine to form a triple helix that can bind up to 3 tetramers of AChE. The C-termini of ColQ are inserted in the basal lamina, an extracellular matrix that ensheaths the postsynaptic muscle membrane (see Chap. 44). Thus the catalytic subunits of AChE protrude into the synaptic cleft, where they are optimally placed to devour ACh.

AChE tetramers are tethered to plasma membranes, notably in brain, via a 20 kDa hydrophobic transmembrane protein called PRiMA (**p**roline-**r**ich **m**embrane **a**nchor; Perrier et al., 2002). PRiMA is linked by disulfide bonds to cysteines in AChE. The extracellular domain of PRiMA has a PRAD motif that could make a coiled-coil interaction with the T-peptides of an AChE tetramer, as observed for ColQ. It is postulated that PRiMA orientates the AChE catalytic subunits optimally for ACh hydrolysis.

AChE is encoded by a single gene that is subject to alternative splicing

How are these various molecular forms of AChE generated? In vertebrates AChE is encoded by one gene comprising 6 exons (Fig. 13-4) but diversity is generated by alternate promoter usage and alternative splicing of nascent mRNA transcripts to give proteins that differ in their N- and/or C-termini (Massoulié, 2002). The catalytic domain is governed by exons 2, 3 and 4 that are transcribed in all forms of AChE. These exons are followed by three splicing alternatives. The first splice site is in exon 4. If this is ignored and translation continues through exon 4 (but omitting exons 5 and 6), a soluble monomeric species (termed 'readthrough' R, Fig. 13-4) results; this is only found at very low levels under normal conditions. Splicing to include only exon 5 gives rise to a C-terminal sequence that contains a cysteine residue for dimerization and a hydrophobic domain for attachment of a GPI anchor. This GPI-anchored, dimeric form of AChE, termed H for 'hydrophobic', is mostly found on blood cells. Splicing to exon 6 (excluding exon 5) encodes the 40 amino acid T-peptide: this is the most common form of AChE expressed in the nervous system and NMJ. This T-peptide contains cysteine residues for covalent assembly as homodimers and homotetramers, and promotes the association of tetramers with the structural subunits ColQ and PRiMA, as described above.

In mammals and birds, a related enzyme termed butyrylcholinesterase is the product of a separate gene. This enzyme exists as a soluble protein in plasma. Although structurally related to AChE and capable of hydrolyzing ACh, its optimal substrates have larger acyl groups, as its name implies (Taylor & Radic, 1994; Massoulié, 2002). The physiological function of butyrylcholinesterase remains unclear but its likely role is the hydrolysis of potentially toxic esters of dietary origin.

AChE catalysis: mechanism of a nearly perfect enzyme

The amino acid sequence of AChE shows no sequence identity with other hydrolytic enzymes but its primary structure is consistent with adopting the same α/β folding pattern common to serine hydrolases such as carboxypeptidase. Serine hydrolases employ a catalytic triad that uses a dicarboxylic amino acid to withdraw a proton from the catalytic serine via the imadazole of histidine, rendering the serine

more nucleophilic (see Fig. 13-5). In AChE from *Torpedo* electric fish, Ser_{200} was identified as the active site serine by labeling it with the irreversible competitive inhibitor diisopropylfluorophosphate (DFP). His_{440} was proposed as a component of the AChE triad following mutagenesis experiments. These amino acids, together with Glu_{327}, were confirmed as constituting the catalytic triad when the crystal structure of *Torpedo* AChE was resolved in 1991 (Sussman & Sillman, 1991) (see below).

The catalytic mechanism proceeds through the following series of steps, illustrated in Fig. 13-5:

1. Ser_{200} makes a nucleophilic attack on the carbonyl carbon of ACh, to form a transient covalent tetrahedral transition state; His_{440} picks up the proton from the serine hydroxyl group.
2. The tetrahedral transition state relaxes back to the trigonal acyl enzyme with rupture of the ester bond, transfer of the proton from His_{440} to the choline oxygen and release of choline.
3. The resultant acetyl–serine intermediate is subject to nucleophilic attack by a water molecule to form a second tetrahedral transition state; the water molecule is likely rendered more nucleophilic by donating a proton to His_{440}.
4. Collapse of the tetrahedral transition state releases acetate and restores the active site serine.

This is a very efficient mechanism; with ACh as substrate, the half-life of the acetyl-serine intermediate is 10μs.

The active site is at the bottom of a narrow gorge in the AChE protein

The crystal structure of AChE was first determined for the enzyme from *Torpedo* (see Fig. 13-8) where it exists as a GPI-anchored dimer and was purified after cleavage from the membrane by phospholipase C. The resultant crystal structure, resolved to 2.8Å, confirmed that the enzyme belongs to the class of α/β proteins and has a catalytic triad mechanism of catalysis (Fig. 13-6), but it also threw up a number of surprises

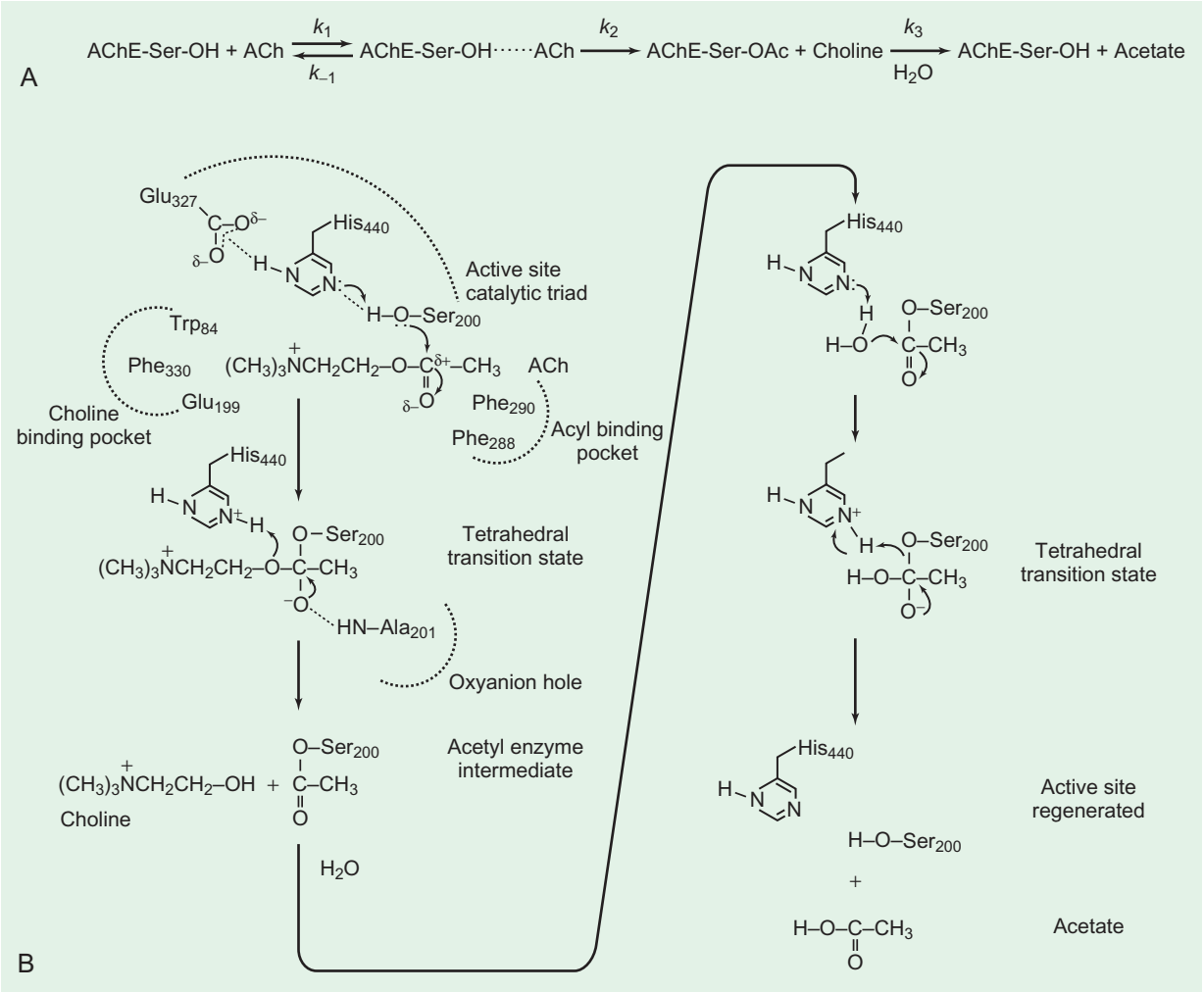


FIGURE 13-5 **Catalytic mechanism of ACh hydrolysis by AChE. (A)** overview; **(B)** catalytic mechanism via tetrahedral transition states and acetyl–serine intermediate. Upper section includes the catalytic triad and residues that stabilize and orientate the substrate. See text for details.

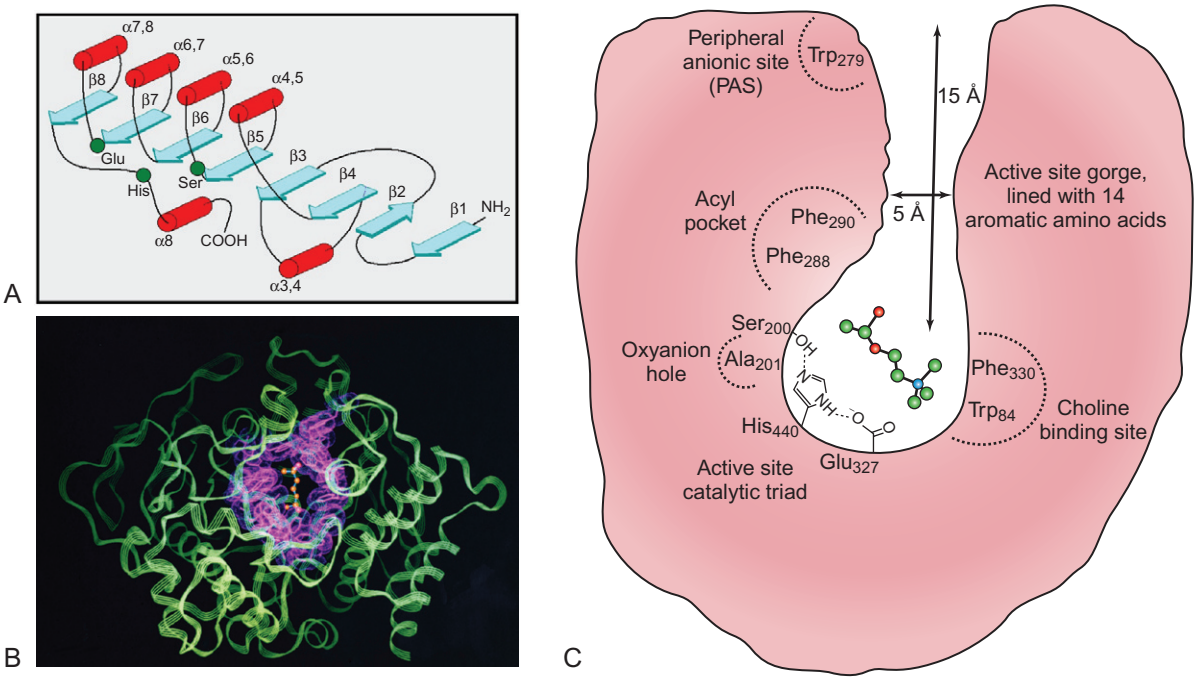


FIGURE 13-6 **The molecular structure of AChE. (A)** Schematic representation of secondary protein structure showing β sheets surrounded by α helices. The relative locations of the catalytic triad residues are indicated. **(B)** Crystal structure of AChE monomer, looking down the gorge (aromatic residues in purple) with ACh inserted in the active site. **(C)** Cartoon of a section through AChE, showing ACh in the active site at the base of the aromatic gorge. A and B from Sussman & Silman (1992).

(Sussman & Silman, 1991). The dicarboxylic amino acid of the triad was identified from its relative position as Glu_{327}; by contrast, aspartic acid has this role in serine proteases and most other hydrolases. Moreover, the triad has the opposite handedness to serine proteases; that means that the orientation of the three amino acids that comprise the triad, in relation to the polypeptide backbone, is the mirror image of their orientation in proteases like chymotrypsin. Together with the lack of amino acid sequence identity, it can be concluded that AChE acquired its catalytic mechanism by convergent evolution.

The most surprising finding was that the active site lies at the bottom of a narrow gorge that extends 20 Å into the enzyme. How is this compatible with its catalytic efficiency? It has been proposed (Silman & Sussman, 2008) that burying the active site enables the substrate to be enveloped by the protein, permitting more interactions to provide greater stabilization of the transition state, resulting in faster catalysis. Thus Trp_{84} and Phe_{330}, together with Glu_{199}, contribute to a choline-binding pocket, whereas Phe_{288} and Phe_{290} constitute an acyl-binding pocket that stabilizes and orientates the acetyl portion of ACh. The tetrahedral transition state is stabilized via a three-pronged interaction with Ala_{201} (see Fig. 13-5).

The rate of ACh catalysis is close to being diffusion limited, so access of ACh to the active site via the gorge must not impose any limitation. The gorge is lined with aromatic amino acids and there is a large dipole moment oriented along the axis of the gorge. This might attract positively charged ACh down to the active site, facilitated by cation-π interactions between the quaternary ammonium of ACh and the aromatic residues. Aromatic amino acids, such as Trp_{279}, also contribute to the so-called peripheral anionic site (PAS) at the mouth of the gorge (see Fig. 13-6). The PAS was first defined in kinetic studies as the site of action of allosteric (noncompetitive) inhibitors of AChE. The PAS weakly binds ACh through π (noncovalent) interactions and is proposed to trap the substrate, enhancing its entry into the gorge (Silman & Sussman, 2008). The importance of the PAS is indicated by the reversible inhibition of AChE activity produced by inhibitors that bind here, including the peptide fasciculin that is found in the venom of the green mamba.

Inhibitors of AChE have toxicological, agrochemical and clinical significance

The importance of AChE is illustrated by the dramatic consequences of its inactivation by organophosphorus agents such as DFP, an inhibitor that generates a very stable phosphorylserine enzyme intermediate (Fig. 13-7), making the enzyme unavailable for the hydrolysis of ACh. This mechanism of inhibition forms the basis of action of nerve gas agents (see Box 13.1). If untreated, death results from respiratory failure due to paralysis of the intercostal and diaphragm muscles necessary for breathing. The prolonged presence of ACh and its extended occupation of muscle nicotinic receptors produce persistent depolarization of the muscle endplate. Such excessive depolarization leads to a diminished capacity to initiate coordinated muscle action potentials. Fasciculations, or muscle twitches, are observed initially, followed by flaccid paralysis. Insects use nicotinic cholinergic transmission for fast excitatory transmission in the insect

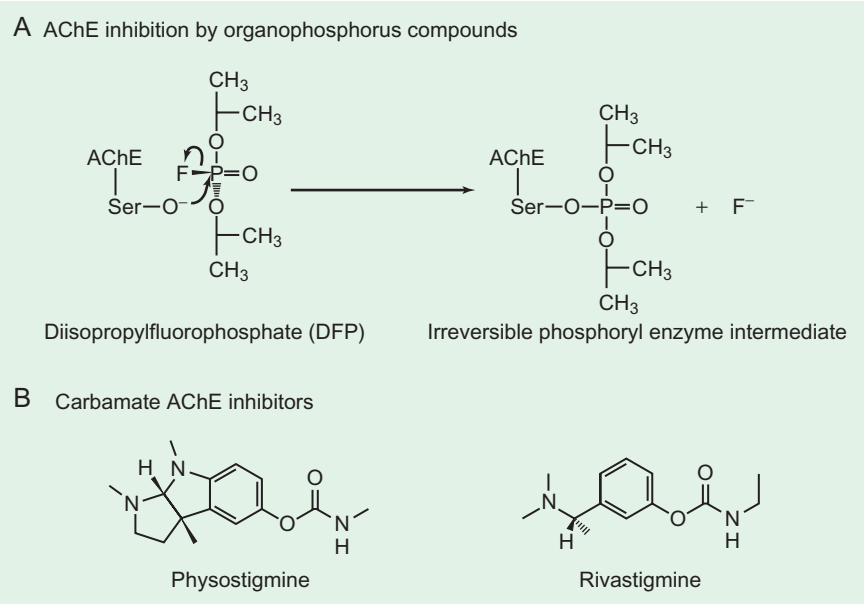


FIGURE 13-7 AChE inhibitors that form longer-lived enzyme intermediates.

'brain,' and this has been targeted by organophosphorus pesticides, which are used to inactivate insect AChE by an analogous mechanism. Accidental organophosphorus poisoning can result from exposure to these agrochemical products.

A less toxic class of AChE inhibitors is the carbamate series (Fig. 13-7), which forms a slowly reversible carbamoylserine enzyme intermediate. Such drugs have been given prophylactically to protect against organophosphate anticholinesterase poisoning (see Box 13.1) and also have clinical applications. They are currently used for the treatment of several conditions with a cholinergic deficit. Myasthenia gravis (see Chap. 44) is characterized by muscle weakness due to an autoimmune attack on the nicotinic receptors at the NMJ. Modest inhibition of AChE enhances the lifetime of released ACh to produce greater nicotinic stimulation. The carbamate physostigmine was first used clinically in the 1930s to treat myasthenia gravis. The eye condition glaucoma has also been treated with physostigmine and other anticholinesterase drugs. They provide symptomatic relief by promoting cholinergic parasympathetic contraction of the ciliary muscle, increasing drainage of aqueous humor and reducing intraocular pressure. A related carbamate inhibitor, rivastigmine, is currently employed to treat mild to moderate cognitive symptoms of Alzheimer's disease (AD; see Chap. 46). Two structurally unrelated, competitive AChE inhibitors, donepezil and galantamine, are also approved for clinical use in AD. In this condition the clinical target is the degenerating cholinergic innervation of the cortex and hippocampus; inhibition of AChE boosts the actions of residual ACh to produce a relatively short-term, modest symptomatic benefit.

Does AChE have other functions?

There has been debate for many years about the possibility that AChE has additional, 'non-classical' functions. The lack of correlation between AChE activity in the CNS and regions of cholinergic innervation, along with the observation that AChE is expressed prior to synaptogenesis, have stimulated enquiry into this possibility (Soreq & Seidman, 2001). Two main additional roles have been proposed:

- *Hydrolysis of ACh that has trophic actions.* In this scenario AChE exerts its catalytic activity to remove ACh acting nonsynaptically in neural tissues or ACh having a trophic or hormonal role in non-neural tissues (e.g., lung).
- *Protein–protein interactions relevant to synaptic development and maintenance*. This proposal is based on the high sequence similarity between AChE and certain neuronal adhesion proteins such as neuroligin that share the same α/β protein fold but lack the catalytic serine characteristic of esterase activity (Soreq & Seidman, 2001; Silman & Sussman, 2005). Laminin-1 has been identified as a binding partner of AChE, which may function as a heterophilic cell adhesion molecule or a third partner in concert with a classical binding pair (Johnson et al., 2008). Laminin-1 has a role in neuronal differentiation and neurite outgrowth, with integrins as its established binding partner (see Chap. 9).

NICOTINIC CHOLINERGIC RECEPTORS

The nicotinic receptor was the first receptor to be characterized biochemically

After the early studies of Langley and Dale that led to the classification of nicotinic acetylcholine receptors (nAChRs; Maehle et al., 2002), muscle nAChRs were extensively investigated using electrophysiological techniques that advanced during in the 1950s. These studies were facilitated by the

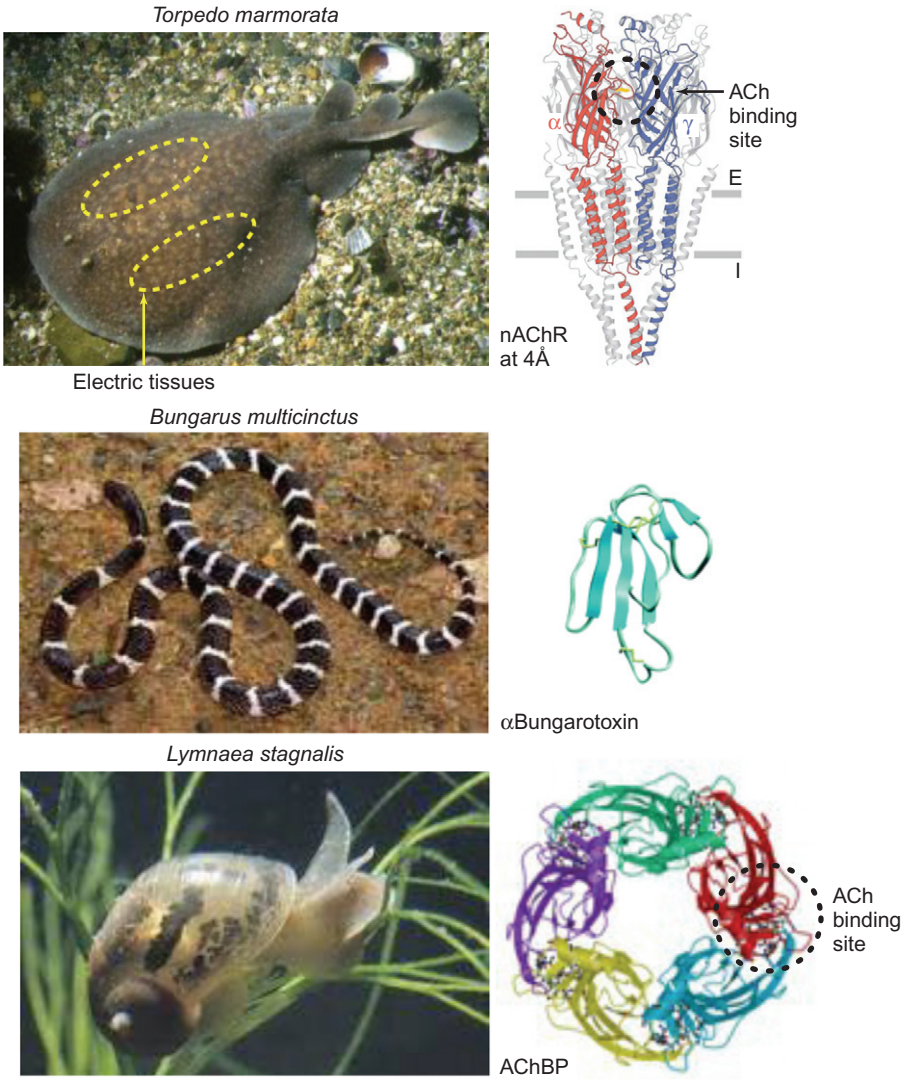


FIGURE 13-8 Animals that have facilitated nAChR research. *Upper panel:* The marine ray *Torpedo marmorata*; a rich source of muscle-type nAChR present in its electric tissues that has allowed detailed structural determinations using electron microscopy. On the right is a ribbon diagram of *Torpedo* nAChR, viewed parallel with the membrane plane. For clarity, only the front two subunits are highlighted: α in red, and γ in blue. Also shown is the location of αtrp149 (gold) at the agonist-binding site. The membrane is indicated by horizontal bars; E, extracellular; I, intracellular. From Unwin, 2005. *Middle panel*: the banded krait *Bungarus multicinctus* is a source of the potent nAChR antagonist α-bungarotoxin, a component of this snake's venom. The 'three-finger' structure of this polypeptide toxin is shown. *Lower panel*: The freshwater snail *Lymnaea stagnalis* yielded the novel AChBP. The crystal structure of this soluble protein provided a detailed molecular description of the agonist-binding site and confirmation of models based on other methods. On the right is a ribbon structure of the AChBP viewed from above. Each of the identical subunits is colored differently for clarity. One ACh-binding region at the subunit interface is indicated. From Brejc, et al., 2001.

relative accessibility and homogeneity of skeletal muscle tissue, and the muscle end plate of the NMJ in particular, compared with the synapses of the central and peripheral nervous systems. In the 1970s, the nAChR was the first receptor to be purified. Two organisms helped to make this possible (Fig. 13-8). First, a rich source of receptor was needed; this was provided by the electric organ of the *Torpedo* ray. The electric tissues of this fish consist of stacks of electrocytes that have the same embryonic origin as skeletal muscle. Instead of developing into contractile muscle cells with very localized endplates where the nAChRs are found, electrocytes express nAChRs over their whole surface in order to maximize the electrical current generated, producing an electrical discharge of ~60 volts and 50 amps of current. The concentration of nAChRs, at 100 pmol/mg protein, is 1000 times higher than in skeletal muscle. Biochemical purification relied on a second factor, a high-affinity ligand to tag and track the nAChR during solubilization and affinity chromatography. This was α-bungarotoxin, a very potent, selective and essentially irreversible antagonist of muscle nAChRs that is found in the venom of certain snakes, notably the banded krait *Bungarus multicinctus* (Fig. 13-8). The biochemical characterization of muscle-type nAChRs from *Torpedo* was complemented by structural studies at a level of resolution unprecedented for receptor proteins in the 1980s. The high density of nAChRs in *Torpedo* electric organ membranes provides a semi-crystalline array that is amenable to high-resolution cryo-electron microscopy techniques for structural determination. The structure of the nAChR has been resolved to 4 Å using this approach (Unwin, 2005), as is discussed further below.

nAChRs are pentameric ligand-gated ion channels

Biochemical studies carried out in the late 1970s enabled purification of the nAChR from *Torpedo*. The receptor was resolved on SDS-polyacrylamide gels as 4 bands of MW between 40 and 65, indicative of four different subunits,

designated α, β, γ and δ in order of increasing MW. The α subunit is present in *Torpedo* and muscle nAChRs in two copies. Thus the nAChR is a pentamer of molecular mass approximately 280 kDa. Subsequently, the cDNAs for these subunits were cloned and sequenced, and then related genes from vertebrate muscle, including human muscle, were obtained using similar approaches (Changeux et al., 1984). Unlike in *Torpedo*, muscle cells express an additional subunit, ε, that replaces the γ subunit in mature (innervated) muscle nAChRs. The nAChR subunits have homologous amino acid sequences with 30–40% amino acid identity, indicative of their evolution by gene duplication of a common ancestor (le Novère & Changeux, 1995). Hydropathy profiles showed each subunit to have four highly conserved hydrophobic regions consistent with four transmembrane domains. Glycosylation of the long N-terminal sequence placed this section extracellularly. Thus each subunit folds as illustrated in Fig. 13-9.

Structural models based on high-resolution EM showed that the subunits span the membrane to create a symmetrical pentameric assembly surrounding a central pore, the ion channel. The order of the subunits around the channel was established to be α, γ, α, δ, β. ACh binding to the extracellular domain leads to opening of the channel to allow the flux of ions. Under normal conditions Na^+ and Ca^{2+} flow into the muscle cell, producing depolarization, whereas K^+ flows out (Karlin, 2002).

Agonists bind at the interface between adjacent subunits

The agonist-binding site was assigned to the α subunits, close to a pair of adjacent, disulfide-bonded cysteine residues ('vicinal' cysteines) in the N-terminal extracellular domain, at positions 190 and 191 in the *Torpedo* sequence. Reducing this disulfide bond or replacing the cysteine residues abolishes agonist activity, whereas reagents such as bromoacetylcholine irreversibly label the vicinal cysteines (Karlin, 2002). Mutagenesis and affinity labeling experiments have identified additional amino acids important for agonist binding. These occur on three noncontiguous loops of the α subunit (the 'principal' component)

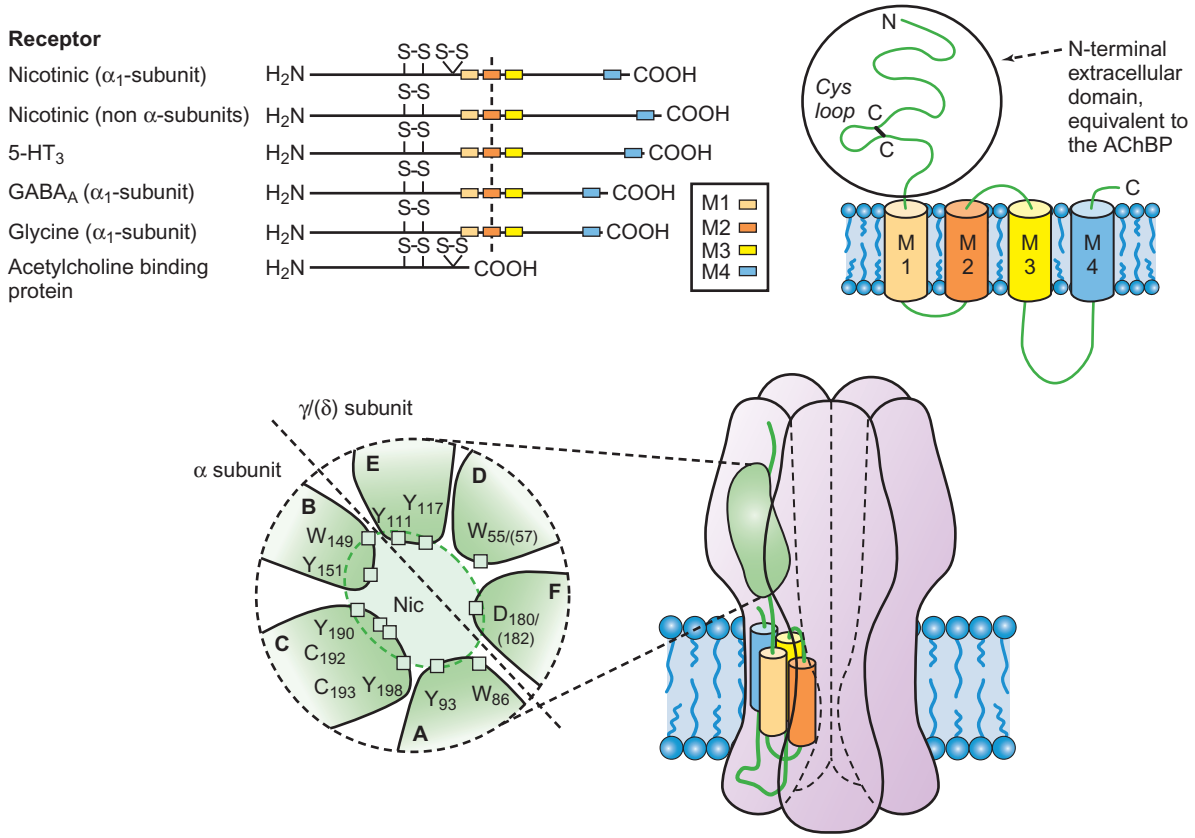


FIGURE 13-9 **Structural features of the nAChR. Top left:** Schematic representation of the sequence of various cys-loop receptor subunits including the AChBP, highlighting key conserved features. Reading from the N terminus, the disulfide-bonded cys loop is common to all these subunits and defines the family. The pair of vicinial cysteines close to the first transmembrane domain is a characteristic of nAChR αsubunits and the AChBP only. The colored boxes represent the four transmembrane segments, M1, M2, M3 and M4. The intracellular loop between M3 and M4 is variable in length. **Top right:** Orientation of a nAChR subunit within the membrane. **Bottom:** schematic of an assembled nAChR, with five subunits arranged to create a central ion channel, lined by M2. The N-terminal ACh-binding site is shown in the insert to be composed of three protein loops from the α subunit (principal face) and three loops from the adjacent (complementary) subunit, in this case the γ subunit of a muscle nAChR. Key amino acid residues involved in ACh binding are indicated.

and on three loops from the adjacent subunit (the 'complementary' component; Fig. 13-9). In the *Torpedo* nAChR, the adjacent subunit differs for each of the α subunits, being either γ or δ. Consequently the two binding sites, formed at the αγ and αδ interfaces, are distinct and exhibit pharmacological and kinetic differences. Kinetic studies indicate that both sites must be occupied for effective opening of the ion channel to occur.

The binding site model has been corroborated by structural studies on a novel protein originally found in the freshwater snail *Lymnaea* (Fig. 13-8). This is a soluble ACh-binding protein (AChBP) that has a homopentameric structure homologous to the pentameric extracellular N-terminal domain of a nAChR (Brejc et al., 2001). In particular, the amino acids of the principal and complementary loops are conserved, indicative of a comparable mode of ACh binding. As a soluble protein, the X-ray crystal structure of the AChBP could be determined relatively easily, in contrast to the membrane-bound nAChR, providing a detailed molecular view (Fig. 13-8). Co-crystallization of the AChBP with agonist (carbamylcholine or nicotine) bound established the interactions between the conserved amino acids of the loops and a nicotinic agonist (Celie et al., 2004). It is noteworthy that most of these conserved amino acids are aromatic, and make cation-π interactions with the agonist, a mechanism reminiscent of the interactions between ACh and AChE. Competitive antagonists, such as α-bungarotoxin, bind to the nAChR in the vicinity of the agonist-binding site, denying access to the agonist ligand.

The nAChR is the prototypical member of the cys-loop family of ligand-gated ion channel receptors

Following the cloning of muscle nAChR subunits, subunits of the $GABA_A$ (Chap. 18), glycine (Chap. 12) and $5HT_3$ (Chap. 15) receptors were cloned and discovered to share homology with nAChR subunits (Fig. 13-9), despite the fact that $GABA_A$ and glycine receptors are ligand-gated chloride ion channels that typically cause hyperpolarization. In addition to a conserved subunit topology and pentameric association to create an ion channel, all the subunits of these receptors and nAChRs share a common motif. This is a 13-amino-acid loop, formed by a disulfide bond between cysteine residues at positions 128 and 142 (*Torpedo* numbering) in the N-terminal extracellular domain. This loop has been implicated in transducing agonist binding into channel opening (Sine & Engel, 2006).

The nAChR ion channel

Binding of ACh to the nAChR is communicated to the ion channel extremely rapidly: electrophysiological measurements show that channel opening occurs within tens of microseconds of ACh application, despite a distance of some 50Å between the binding site and the channel (Sine & Engel, 2006). The nAChR channel is made up of the second transmembrane segments (M2) from each subunit (Fig. 13-9). In the resting (closed) state, the narrowest part of the channel has a diameter of about 6Å, too narrow for hydrated ions to pass through. This constriction or 'gate' has been identified in the lower (closer to the cytoplasmic side) half of the channel. In some elegant experiments, the 'open' nAChR was captured by rapid freezing of *Torpedo* membranes within 5ms of applying ACh. This allowed the open state of the channel to be examined by electron microscopy. This and other studies have indicated that binding of ACh results in a conformational change in the extracellular domain that promotes a 15° twist of the M2 αhelices and widening of the pore by 3Å at its narrowest point, sufficient for the passage of ions (Unwin, 2005). Ion flux through the channel is believed to be facilitated by interactions with the side chains of amino acids lining the channel.

The sequential binding of two ACh molecules (or agonist, A) to the muscle nAChR (R) results in channel opening; this can be described by the following scheme:

$$\underset{\text{(Closed)}}{2A + R} \rightleftarrows \underset{\text{(Closed)}}{AR} \rightleftarrows \underset{\text{(Closed)}}{A_2R} \rightleftarrows \underset{\text{(Open)}}{A_2R^*}$$

For ACh, the forward rate constant (k_1) for binding to the nAChR to give A_2R is 1–2 × $10^8 M^{-1}s^{-1}$. The forward and reverse rate constants k_2 and k_{-2} for the isomerization to A_2R^* (the conformational changes leading to channel opening and closing) yield rates of isomerization consistent with opening events in the millisecond timescale. Transitions between states occur randomly and as k_2 and k_{-2} are greater than k_{-1} (the rate constant for ligand dissociation), several opening and closing events can occur before the ligand dissociates (Colquhoun et al., 1987). The efficacy and duration of channel opening is dependent on the particular agonist.

The prolonged presence of agonist leads to desensitization

ACh is normally hydrolyzed rapidly by AChE. However, if AChE is inhibited, or exogenous drugs such as nicotine (that are not metabolized locally) are applied, the responsiveness of the nAChR diminishes over time, despite the sustained presence of agonist. This phenomenon is referred to as receptor desensitization, and at least two desensitized states can be distinguished kinetically: fast-onset and slow-onset desensitized. A simplified scheme is shown in Fig. 13-10. (In reality, this is much more complex, because each liganded state in Scheme 1 is in equilibrium with its corresponding desensitized states.) Note that agonist remains bound in the desensitized states. In physical terms, the desensitized states represent distinct conformations of the nAChR that differ from either the resting (closed, in the absence of agonist) or open configurations. Receptor desensitization presumably occurs as a protective mechanism to prevent over-excitation of the target cell.

Neuronal nAChRs form a family of related receptors

Much of the work defining the fundamental principles of nAChR structure and mechanism has been carried out on

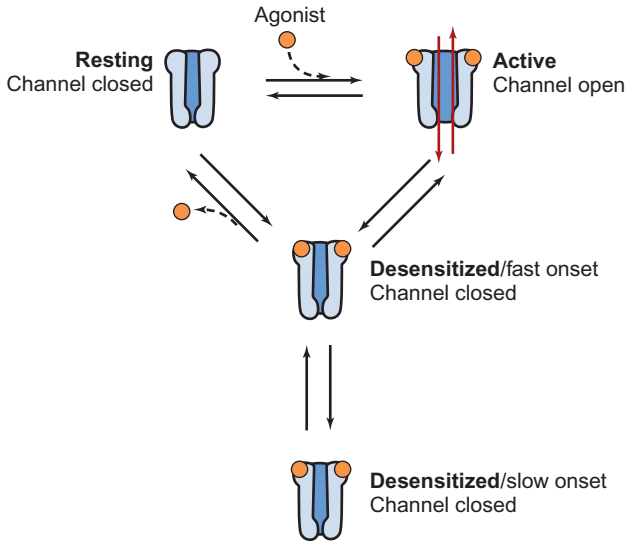


FIGURE 13-10 **Conformational states of a nAChR.** In the resting state the channel is closed with low probability of opening. ACh or agonist binding greatly increases the probability of opening, leading to ion flux through the channel. Despite the continued presence of bound agonist, the open state is not maintained and the nAChR adopts a closed conformation that is unresponsive to agonist. The nAChR is said to be desensitized. Multiple desensitized states can occur; two states are shown here.

muscle or *Torpedo* nAChRs, as described above. However, it had been known for a century that nAChRs are also present in autonomic neurons, where they show some pharmacological differences from nAChRs in muscle (Paton & Zaimis, 1949). nAChRs in the brain were predicted when it was recognized that nicotine dependence is responsible for addiction to tobacco smoking. Cloning of the first neuronal nAChR subunits was published in 1987 and led to the discovery of a plethora of subunits; the tally now stands at 11 neuronal nAChR subunits in mammals with an additional one (α8) in avian species (Fig. 13-11; Millar & Gotti, 2009). These share all the features characteristic of cys-loop receptors (Fig. 13-9). None of the muscle subunits are expressed in neurons.

Neuronal nAChR subunits are classified as 'α' if they also contain the vicinal cysteines required for agonist binding. The muscle α subunit is designated α1, with α2-α10 being neuronal subunits. However, α5 lacks certain amino acids from the binding site loops, so it is not a true α subunit capable of contributing to the principal face of a binding site. The α4 subunit is almost exclusively found in CNS neurons, whereas α9 and α10 are not expressed in the CNS but are located in cochlear hair cells and some sensory neurons. Subunits lacking the vicinal cysteines are designated 'β', with β1 contributing to muscle nAChR and β2-β4 expressed in various nonmuscle cell types.

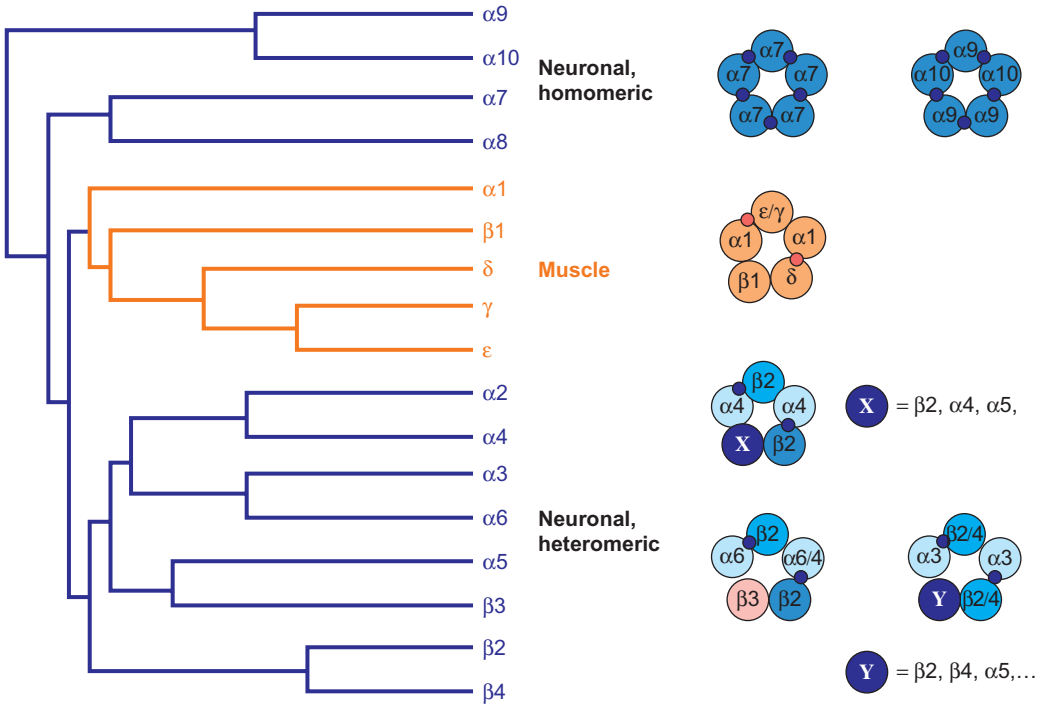


FIGURE 13-11 **Heterogeneity of nAChR family of subunits.** Phylogenetic relationship of vertebrate nAChR subunits, adapted from Le Novère & Changeux (1995). The subunit composition of native nAChRs is illustrated on the right. Putative agonist-binding sites are indicated by dark circles between principal and complimentary adjacent subunits.

The permutations of subunits forming nAChRs create more diversity

The assembly of neuronal nAChR subunits to form pentameric receptors is not fully understood. The α7 subunit is unique in existing predominantly in homopentameric nAChRs in mammalian cells. This appears to reflect its evolutionary proximity to the ancestral gene, compared with other nAChR subunits (Fig. 13-11). Indeed, some nAChR subunits discovered in insects and *C. elegans*, as well as the AChBP, are more closely related to the α7 nAChR than other vertebrate subunits (Le Novère & Changeux, 1995). α7 nAChRs have some distinctive properties, including very high permeability to Ca^{2+} and a propensity to desensitize rapidly in response to agonist, that have made them difficult to study. The recent development of positive allosteric modulators (PAMs) that can amplify agonist responses by shifting the agonist dose-response relationship to the left and, in some cases, preventing or reversing desensitization, has provided a useful tool for studying the function of α7 nAChRs (Gronlien et al., 2007). These receptors are also sensitive to αbungarotoxin, in contrast to the α/β heteromers described below.

α9 can also form homopentamers in *in vitro* systems, but it probably associates with the α10 subunit in native receptors (Vetter et al., 2007). All of the other mammalian neuronal α subunits (except α5) combine with β subunits to form heteromeric nAChRs with at least 2 α subunits, thus generating two agonist-binding sites at the α/β subunit interfaces (Millar & Gotti, 2009). For example, α3 and β4 are the major subunits constituting the ganglionic nAChR in autonomic neurons. α4 and β2 are the most abundant nAChR subunits expressed in the brain and combine to form a nAChR with high affinity for nicotine and ACh. Recently it has been shown that nAChRs with the stoichiometry $(\alpha4)_2(\beta2)_3$ have higher affinity for ACh than those with stoichiometry $(\alpha4)_3(\beta2)_2$, even though both types have two identical agonist-binding sites at the α/β interfaces. As exposure to nicotine promotes conversion to the higher-affinity form, subunit switching may have a physiological role.

Subunit assemblies can get more complicated, with multiple types of α and/or β subunit in a single receptor (Fig. 13-11). α5 and β3 subunits behave like the muscle nAChR β subunit in terms of the rules of assembly, and do not contribute directly to the agonist-binding sites but constitute the fifth subunit in the pentamer. These are referred to as 'accessory subunits' (Kuryatov et al., 2008). Nevertheless, the presence or absence of an α5 subunit, in an α4β2 nAChR for example, influences properties of the receptor such as sensitivity to ACh and Ca^{2+} permeability. The α5 subunit has also been proposed to confer a binding site for allosteric modulators (Kuryatov et al., 2008), and in this respect resembles the γ subunit of $GABA_A$ receptors (see Chap. 18). Because the exact subunit composition of a native nAChR is often unknown, an asterisk is used to indicate that additional subunits may be present. For example, α4β2* nAChR could also include α5 or α6 or β3 etc.

Neuronal nAChRs modulate brain function

The expression of nAChR subunits in the brain is not uniform. α4 and β2 subunits are relatively abundant in cortex, thalamus and dopamine neurons, whereas the α7 subunit is highly expressed in hippocampus. In addition, the less common α2, α3, α5, α6, β3 and β4 subunits are found in various brain nuclei. For example, expression of α6 and β3 subunits is mostly restricted to catecholaminergic neurons. The functional effects of nAChRs in the brain were difficult to elucidate: by analogy with the NMJ and autonomic ganglia it was assumed that these ligand-gated cation channels would mediate fast synaptic transmission, but such responses are uncommon and nAChRs mostly exert a more subtle, modulatory influence. Many subtypes are located presynaptically on nerve terminals where they can influence transmitter release. Presynaptic nAChRs and some somatodendritic nAChRs respond to ACh that is released at a distance and exerts its influence by volume transmission. Detailed studies of dopamine neurons have revealed multiple different α4β2* nAChRs on both cell bodies and terminals (Livingstone et al., 2009). The ability of nicotine to promote dopamine release by activating (or desensitizing) these nAChRs is considered critical for the reinforcing properties of nicotine that produce dependence (see Chap. 61).

α7 nAChRs are more commonly associated with glutamate terminals and GABA neurons. They contribute to synaptic plasticity, including LTP, by virtue of their high Ca^{2+} permeability and activation of Ca^{2+}-dependent processes. These include short-term local signaling events governed by kinases and phosphatases, as well as long-term changes mediated by transcriptional regulators (Dajas-Bailador & Wonnacott, 2004). A role in development has also been proposed for α7 nAChRs as they are first expressed before the formation of cholinergic synapses; they appear to assist in the switch in GABA signaling, from excitatory during development to inhibitory once synapses are formed (Chap. 18) (Liu et al., 2007).

Transgenic mice help to reveal the physiological roles and clinical implications of nAChRs

Transgenic mice deficient in each one of the neuronal nAChR subunits have been generated (Champtiaux & Changeux, 2004). All survive and have no overt phenotype, with one exception: a null mutation of the α3 subunit produces a number of defects consistent with its role in the autonomic control of key physiological processes such as bladder function. Homozygous α3-null mutant animals tend to die within the first week after birth (Xu et al., 1999a). It is surprising to find that neither β4 nor β2 knockout mice show such defects, when one of these β subunits is essential for forming a nAChR with α3. However, the double β2/β4 knockout results in mice with autonomic dysfunction and death in the first weeks after birth (Xu et al., 1999b). Thus β2 and β4 can compensate for one another, a dramatic example of the adaptability in biological systems and the caution necessary when interpreting results from transgenic manipulations.

The brains of β2- (but not β4-) null mutant mice lack high-affinity binding of nicotine, and these animals fail to self-administer nicotine, a model of voluntary drug taking associated with addictive drugs (Picciotto et al., 1998). They

also fail to release dopamine in response to nicotine. These observations, together with evidence from α4 knockout animals, make α4β2* nAChRs of prime importance for nicotine addiction, and a primary target for smoking cessation drugs (Changeux, 2010).

Neuronal nAChRs are also present in non-neuronal cells

There is increasing recognition of a non-neuronal cholinergic system in which ACh is synthesized and released by non-neuronal cells to exert functions mediated by nicotinic and muscarinic receptors on such cells, while the effects of ACh are terminated by hydrolysis by AChE (Wessler & Kirkpatrick, 2008). nAChRs have attracted particular attention in epithelial cells (especially in the airways) and immune cells. Several studies have reported the expression of most nAChR subunits (including those from muscle nAChRs) in such cells, but most attention has focused on α7 and 'non-α7' neuronal nAChRs. Their functions in these cells remain controversial but include possible roles in inflammation and in cell growth, maturation and proliferation during development. The latter has raised the specter of procancerous actions, particularly with respect to nicotine, which could promote tumor growth in smokers.

nAChRs and disease

There are several examples of nAChRs having a causal or contributory role in diseases, due to genetic mutations or polymorphisms, or autoimmune attack.

1. Autoimmune diseases. Myasthenia gravis is characterized by the presence of circulating autoantibodies against the muscle nAChR (see Chap. 44). These antibodies mainly target the α1 subunit, binding to a sequence known as the 'main immunogenic region' (MIR) in the N-terminal extracellular domain. Antibody binding results in removal of nAChRs from the muscle endplate, and muscle weakness is the consequence. Neuronal nAChR subunits have been implicated in other autoimmune diseases (Lindstrom, 2002): antibodies against the α3 subunit occur in some disautonomias, a condition characterized by autonomic dysfunctions such as constipation and dry mouth. The α9 nAChR subunit expressed in keratinocytes is the target of autoantibodies in some patients with pemphigus, a skin condition characterized by blistering.
2. Monogenic disorders. Rare, recessively inherited congenital myasthenic syndromes can result from mutations in the subunits of the muscle nAChR, notably the ε subunit, which replaces the γ subunit around the time of birth in humans. The mutations typically decrease nAChR function. A null mutation in the ϵ subunit has also been characterized. In this case muscle weakness and fatigue results from a reduction in the number of nAChRs at the muscle endplate. Interestingly, the nAChRs are not completely missing, due to rescue by low levels of residual γ subunit that substitutes for the absent ε subunit (Cossins et al., 2004). In mice engineered to have the same null mutation in their ε subunit, this rescue does not occur and the animals die soon after birth when the γ to ϵ switch occurs.
   Mutations in α4 and β2 subunits can result in a rare, inherited form of epilepsy: autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). This condition mostly affects children or adolescents and is characterized by attacks of brief motor seizures during light sleep (Steinlein & Bertrand, 2008; see Chap. 40). The mutations occur in, or close to, the transmembrane sections of the nAChR subunits and appear to alter sensitivity to ACh. How this results in partial epileptic seizures is unclear but may reflect the role of α4β2* nAChRs in modulating GABA release.
3. nAChR polymorphisms. Naturally occurring genetic variation in neuronal nAChR subunits can contribute to the risk factors for neurological and psychiatric diseases and tobacco addiction (Steinlein & Bertrand, 2008). A body of evidence links α7 nAChRs with schizophrenia (Leonard et al., 2007). There is linkage between the α7 gene locus on chromosome 15 and auditory gating deficits common in schizophrenic patients. It is believed that excessive tobacco smoking, which is prevalent among individuals with schizophrenia, is an attempt at self-medication. (More than 80% of individuals diagnosed with the disease are smokers, compared with ~25% in the general population). Clinical trials suggest these sensory deficits can be ameliorated by more selective nAChR agonists that target the α7 nAChR. Polymorphisms in the promoter region of the α7 gene that reduce its transcription appear to be associated with the disease, and this observation is consistent with the reported decrease in the number of α7 nAChRs in postmortem brain samples from schizophrenic patients. Lower levels of α7 nAChRs could contribute to developmental or functional abnormalities believed to underlie the condition (see further discussion in Chap. 58).
   A number of genetic screens have reported linkage between a section of the genome that includes a cluster of nAChR genes also on chromosome 15 and coding for α3, β4 and α5 subunits, with nicotine dependence and smoking-related diseases, notably lung cancer and chronic obstructive pulmonary disease (COPD; Bierut, 2010). Although the risk associated with a polymorphism in this region is very small, indicating that it is but one of a host of factors that must predispose to addiction or lung disease, it is the strongest genetic association found for smoking to date. Of particular interest is a polymorphism in the coding region of the gene encoding the α5 subunit. This is a nonsynonymous substitution that replaces an aspartic acid residue with an asparagine at position 398, located within the M2 transmembrane segment that lines the ion channel. We have already noted that inclusion of α5 in a nAChR alters the properties of the receptor, so this amino acid change might also alter the responsiveness of the nAChR. Indeed, a decrease in response, with no change in agonist sensitivity, was observed when this mutation was recapitulated in an *in vitro* expression system (Bierut et al., 2008). Given the evidence from transgenic mice and other experimental studies for the importance of α4 and β2 nAChR subunits for nicotine dependence (discussed above), the emergence of a different cluster of genes in

the human screens is intriguing. However, α5 is known to assemble with α4 and β2 subunits (Fig. 13-11). With respect to lung disease, α3, β4 and α5 nAChR subunits are expressed in lung epithelial cells, although it is controversial whether the polymorphisms influence lung disease directly or as a consequence of enhancing nicotine dependence.

nAChRs as therapeutic targets

nAChRs are also of interest as therapeutic targets for treating a variety of medical conditions.

1. *Muscle relaxants*. The ability of reversible antagonists of muscle nAChRs to produce muscle relaxation has led to their use as adjuncts to anesthesia in surgery for around 100 years. Paralysis of reflex muscle activity by these agents allows lower and safer doses of anesthetic to be used to produce loss of consciousness (Bowman, 2006). The prototype of this class of drug is the curare alkaloid (+)-tubocurarine (see Fig. 13.1 for structure), a constituent of the arrow poison used by South American Indians. Subsequently synthetic muscle relaxant drugs were developed to optimize their desirable properties (non-depolarizing blockade of muscle nAChRs and more rapid reversal) while minimizing side effects. Examples are pancuronium and vercuronium, which incorporate ACh moieties in their structure to enhance affinity for the muscle nAChR.
2. *Cognitive dysfunction.* Both α7 and α4β2 nAChRs are under consideration as drug targets for treating cognitive decline in Alzheimer's disease and schizophrenia, and also for treating attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders. This is based on the role of the basal forebrain cholinergic system in cortical processing including learning and memory, and the positive effects of nicotine on cognitive performance, especially attention (Hasselmo et al., 2010). Both selective agonists and allosteric potentiators of nAChRs are in preclinical development, with the aim of boosting cholinergic signaling. Allosteric potentiators are ineffective in the absence of agonist and so could be used to boost the effects of endogenous ACh, giving more temporal and regional specificity than can be achieved by delivering an agonist that acts independently of ACh.
3. *Parkinson's disease*. Parkinson's disease is characterized by motor dysfunction that results from the progressive degeneration of the nigrostriatal dopamine pathway. Based on the evidence that smokers are less likely to get Parkinson's disease (even after taking account of the shorter average life span of smokers), nicotinic agonists could be beneficial for treating the disease (Quik et al., 2007). Nicotine could affect disease progression by acting as a neuroprotective agent or could give symptomatic benefit by promoting dopamine release via the nAChRs on dopamine neurons. To target the nAChR subtypes present on these neurons, agonists with selectivity for α4β2* and/or α6β2* nAChRs are being sought. In animal models, nicotine also appears to alleviate drug-induced dyskinesias, the undesirable side effect of standard parkinsonian medication (Bordia et al., 2008; see Chap. 46).
4. *nAChRs as targets for pain relief.* The prospect of nAChR ligands having efficacy for treating pain emerged with the discovery of epibatidine (see Fig. 13.1 for structure), secreted by a South American frog. Epibatidine is an exceptionally high-affinity agonist at α4β2* nAChRs and has exhibited anti-nociceptive properties better than those of morphine (Badio & Daly, 1994). Unfortunately its therapeutic window (before side effects and death occur) is too narrow for clinical applications. Epibatidine has stimulated some intense research into finding alternative nicotinic analgesic drugs. There is current interest in antagonists from another of nature's curiosities: conotoxins secreted in the venom of cone snails, poisonous mollusks that live in subtropical waters such as the Great Barrier Reef. αConotoxins target nAChRs, and variation in the amino acid sequence of these peptide toxins produces remarkable nAChR subtype selectivity. In particular, αconotoxins selective for nAChRs composed of α9 or α9/α10 subunits produce long-lasting analgesia in cases of chronic pain (McIntosh et al., 2009). The α9* nAChR occurs on sensory neurons that transduce pain signals to the spinal cord and presents a novel therapeutic target.
5. *Nicotine dependence.* Addiction to tobacco smoking is the most prevalent condition in which nAChRs are intimately associated. The World Health Organization predicts that half of all lifelong smokers die prematurely due to smoking-related diseases (notably lung cancer and cardiovascular disease); this amounts to 1 in 10 of all adults worldwide. There is a body of evidence to support the view that tobacco addiction is sustained by a chemical dependence on nicotine. While nicotine is not itself particularly harmful (except at high doses), it sustains exposure to the harmful carcinogens and carbon monoxide in tobacco smoke. Nicotine interacts predominantly with α4β2* nAChRs to produce dependence, as discussed above. Indeed, smokers have an increased number of these receptors in their brains. This upregulation can be reproduced in laboratory animals given nicotine for just a few days, and even occurs in cultured cells exposed to nicotine or nicotinic agonists. This paradoxical response (chronic exposure to agonist typically results in desensitization and receptor downregulation, as shown by mAChRs; see below) has challenged researchers for many years, especially because it is a post-transcriptional effect that does not involve increased levels of mRNA (Govind et al., 2009). Recent studies suggest that nicotine, which freely crosses cell membranes, can act as a chaperone to enhance nAChR subunit maturation and assembly, but additional influences on nAChR turnover may also occur. Recently varenicline (see Fig. 13-1 for structure) was launched as an aid to smoking cessation. Varenicline, marketed as Chantix in the United States, is a partial agonist at α4β2* nAChRs (Rollema et al., 2007). A partial agonist produces only a partial functional response despite fully occupying the receptor-binding sites. Thus it is proposed that varenicline mimics nicotine to a limited extent, minimizing withdrawal effects, while preventing nicotine from exerting any additional action if a cigarette

is smoked. Varenicline is the first new drug targeted at a neuronal nAChR to succeed in clinical trials and to reach the clinic.

MUSCARINIC CHOLINERGIC RECEPTORS

Some effects of ACh can be mimicked by the alkaloid muscarine

Early studies by Langley and Dale demonstrated that some of the effects of peripherally administered ACh could be mimicked by muscarine, an alkaloid found in the fly agaric mushroom *Amanita muscaria*. Muscarinic cholinergic responses could be readily distinguished from those elicited by nicotinic agonists in that the muscarinic responses could be either excitatory or inhibitory (nicotinic responses are always excitatory) and could be blocked by the inclusion of submicromolar concentrations of two alkaloids, scopolamine or atropine. Moreover, the responses elicited by muscarinic cholinergic ligands had much longer latencies of both onset (~100–250 ms) and offset than nicotinic responses. These observations led to the concept that separate populations of nAChRs and mAChRs mediate the physiological effects of ACh. mAChRs are widely distributed and are present in neurons (and glia) in most regions of the CNS and the PNS as well as in parasympathetically innervated cardiac and smooth muscles (e.g., heart, iris, stomach, bronchioles, intestines, bladder) and salivary, tear and sweat glands (Table 13-1).

Muscarinic cholinergic responses are mediated by G-protein–coupled receptors

Although the presence of mAChRs in neural and non-neural tissues had previously been inferred, an ability to directly monitor and to quantify these receptors was not feasible until the 1970s, when cholinergic radioligands first became available. Studies in which tissue homogenates or intact cells were incubated with either radiolabeled agonists (ACh or oxotremorine-M) or antagonists (quinuclidinyl benzilate, pirenzepine or N-methylscopolamine) revealed that these ligands bound to mAChR sites in both a saturable manner and with a high degree of specificity. However, whereas muscarinic antagonists bound to the mAChR with a Hill slope of unity, muscarinic agonists such as ACh, carbachol or oxotremorine-M bound to the receptor in a more complex fashion, with evidence for populations of receptors that exhibited low, high or very high affinity for agonists (Birdsall et al., 1978). In the presence of GTP, the affinity of the agonist for the mAChR was dramatically lowered, a finding consistent with the coupling of mAChRs to their effector mechanisms via intermediary GTP-binding proteins (G-proteins), as had previously been observed for β-adrenergic receptors (see Chap. 21). In contrast, the addition of GTP had little or no effect on antagonist binding to mAChR-binding sites. Muscarinic agonists differ in terms of their ability to induce the high- and very-high-affinity binding states of the mAChR. Thus, an agonist such as oxotremorine-M can induce the appearance of a large proportion of high-affinity agonist-binding sites, whereas other muscarinic agonists, such as oxotremorine or pilocarpine, reveal relatively few such sites. The ability of an agonist to induce the appearance of the high-affinity form of the mAChR is predictive of its efficacy. Thus whereas ACh and oxotremorine-M are full agonists for stimulated inositol lipid hydrolysis, pilocarpine and oxotremorine are partial agonists (Fisher, 1986).

Pharmacological studies were the first to indicate the presence of multiple mAChR subtypes

The existence of multiple mAChR subtypes was first proposed on the basis that some compounds appeared to be able to selectively antagonize specific muscarinic responses (see

TABLE 13-1 Physiological Consequences of mAChR Activation in Peripheral Tissues

Tissue	Effects of ACh
Vasculature (endothelial cells)	Release of endothelium-derived relaxing factor (nitric oxide) and vasodilation
Eye iris (pupillae sphincter muscle)	Contraction and miosis
Ciliary muscle	Contraction and accommodation of lens to near vision
Salivary glands and lacrimal glands	Secretion—thin and watery
Bronchii	Constriction, increased secretions
Heart	Bradycardia, decreased conduction (atrioventricular block at high doses), small negative inotropic action
Gastrointestinal tract	Increased tone, increased gastrointestinal secretions, relaxation at sphincters
Urinary bladder	Contraction of detrusor muscle, relaxation of the sphincter
Sweat glands	Diaphoresis
Reproductive tract, male	Erection
Uterus	Variable, dependent on hormonal influence

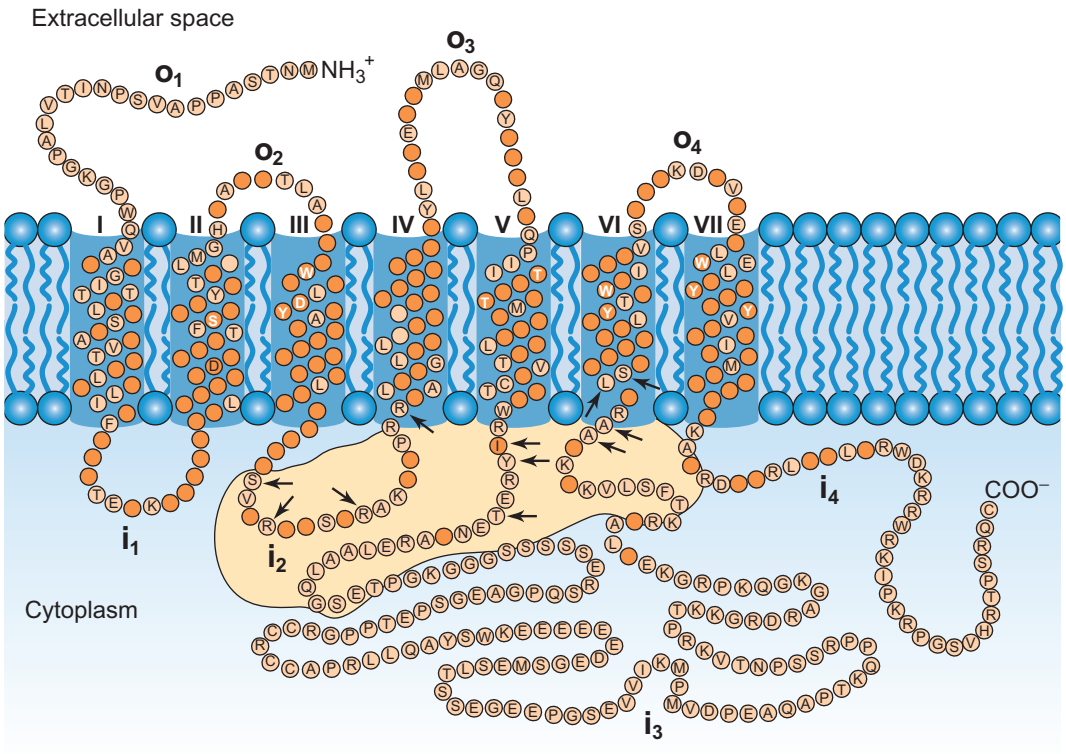


FIGURE 13-12 **Predicted amino acid sequence and transmembrane domain structure of the human M1 muscarinic receptor.** Amino acids that are identical among the m_1, m_2, m_3 and m_4 receptors are *dark orange*. The *shaded cloud* represents the approximate region that determines receptor–G-protein coupling. *Arrows* denote amino acids important for specifying G protein coupling. Amino acids predicted to be involved in agonist or antagonist binding are denoted by *white letters*.

Fig. 13-1 for structures). Chief of these was the cholinergic antagonist pirenzepine, an agent that appeared to selectively block neuronal mAChRs while exhibiting a much lower affinity for receptors in the heart and smooth muscles. On the basis of these results, the pirenzepine-sensitive neuronal receptor was labeled the M1 subtype whereas the non-neuronal, pirenzepine-insensitive form of the mAChR was labeled "M2." Subsequently these M2 receptors could be further subdivided based upon the ability of the pirenzepine analog AF-DX 116 to bind with high affinity to cardiac mAChRs but with low affinity to receptors in the ileum and secretory glands. In contrast, the muscarinic antagonists, hexahydrosiladifenidol (HHSiD) and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), bound with high affinity to mAChRs in the ileum and secretory glands but with low affinity to the cardiac receptors. On the basis of these observations, mAChRs in the heart were labeled M2, whereas those in the ileum and secretory glands were designated as M3. Evidence for an additional subtype (M4) in the striatum was obtained from data in which the mAChR-binding sites in this region exhibited similar properties to those of the M3 receptor in terms of their affinity for pirenzepine, AF-DX 116, HHSiD and 4-DAMP, but (unlike the M3 receptor) exhibited a high affinity for methoctramine. A fifth subtype (M5), revealed from cloning studies, has no distinct pharmacological profile that differentiates it from the other four subtypes.

Molecular cloning of the mAChR reveals five subtypes

Numa and co-workers were the first to isolate the cDNA encoding the brain and heart mAChRs, designated as m1 and m2 respectively, from rat brain and heart cDNA libraries (Kubo et al., 1986). Subsequently, three additional subtypes, termed m3, m4 and m5, were cloned and expressed. The proteins encoded by genes m1-m5 correspond to the receptors pharmacologically identified as M1–M5. The cDNAs for the mAChRs encode proteins of 55–70 kDa and possess seven predicted transmembrane spanning domains, as occurs for the β-adrenergic receptor and other G-protein–coupled receptors (Fig. 13-12). There are also N-glycosylation sites at the N-terminus region and a large cytoplasmic loop between transmembrane domains V and Vl (i_3). The transmembrane segments of the receptor exhibit considerable sequence homology and are packed tightly together. Ligands bind at a site located deep within the bundle formed by transmembrane domains lll–Vll. Of particular importance is

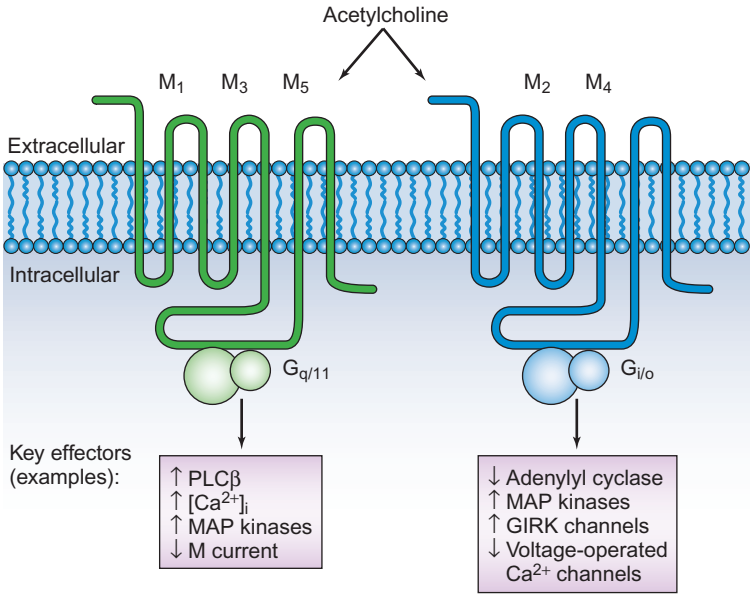


FIGURE 13-13 Muscarinic cholinergic receptors can be subdivided based upon their G-protein–coupling characteristics and effector mechanisms. M1, M3 and M5 mAChRs preferentially couple to G-proteins of the Gq/G11 family, whereas M2 and M4 receptors typically activate G-proteins of the Gi/Go family. Agonist occupancy of the two groups of mAChRs results in the activation of different downstream effector proteins, as indicated, although some effectors (e.g., mitogen-activated protein kinase) (MAPK) are activated by both groups of receptors. Note that the effects of mAChR activation are mediated by both the α and $\beta\gamma$ subunits of the G-proteins (see Chap. 21). An increase or decrease in the activity of the effector mechanism is indicated by the direction of the arrow. GIRK, G-protein–activated inwardly rectifying K^+ channel; PLCβ, phosphoinositide-specific phospholipase C. (Figure adapted from Wess et al., 2007).

an aspartate residue, located in transmembrane domain lll (D-147), which is conserved not only across all mAChRs, but also across all biogenic amine GPCRs. This negatively charged amino acid is essential for interaction with the positively charged ammonium headgroup of the cholinergic ligand (Wess, 1996).

The use of chimeric mAChRs has revealed that the i3 loop is an important determinant of the specificity with which the mAChR subtype couples to individual G-proteins. Site-directed mutagenesis studies have indicated that non-charged amino acids at the N-terminal region of the i_3 loop are required for G-protein recognition and activation, while conserved amino acids in the carboxyl region of the i_3 loop and in the adjacent region of transmembrane domain Vl permit the differentiation of G_i- and G_q-mediated responses (see Chap. 21). There are also phosphorylation sites on the i_3 loop and the carboxyl terminal region of the molecule. These play a role in the internalization and desensitization of the mAChR.

Muscarinic receptor subtypes couple to distinct G-proteins and activate different effector mechanisms

Expression of cloned mAChRs into Chinese hamster ovary cells has demonstrated that mAChRs differ considerably in their ability to activate individual G-proteins and in the ensuing downstream effector mechanisms (see Fig. 13-13). The M1, M3 and M5 mAChRs preferentially couple to G-proteins of the Gq/11 family, which, via either α or $\beta\gamma$ subunits, can increase the activity of phosphoinositide-specific phospholipase C (PLC) with the attendant formation of inositol 1,4,5 trisphosphate and diacylglycerol. These second messengers are responsible for the mobilization of intracellular Ca^{2+} and activation of protein kinase C (PKC, see Chap. 23) and subsequently, that of mitogen-activated protein kinase (MAPK). M1 and M3 mAChRs are also known to promote cell proliferation. M1 receptors have also been shown to inhibit a voltage-sensitive current known as M-current ("M" for muscarinic). mAChR–mediated inhibition of K^+ efflux through the M-channels results in the slow depolarization of the cell and a facilitation of repetitive cell firing.

One of the major consequences of the activation of either M2 or M4 receptors is the negative regulation of adenylyl cyclase activity, an effect mediated by the release of the α_i subunit from pertussis–sensitive G_i. The reduction in cyclic AMP production results in a decrease in the activity of protein kinase A (see Chap. 22). M2 and M4 mAChRs can also cause a rapid activation of G-protein-coupled, inwardly rectifying K^+ channels (GIRKs). However, activation of these channels, which results in membrane hyperpolarization, is a result of the direct interaction of the $\beta\gamma$ subunits with the channel itself; no second messenger formation is required. M2 and M4 receptors can also negatively modulate Ca^{2+} currents whereas they activate MAPK.

Following persistent agonist activation of mAChRs, desensitization of the receptor occurs by means of three kinetically distinct events, as is observed for other GPCRs e.g., β-adrenergic receptor. Initially (within seconds), the mAChR may be phosphorylated by specific kinases, thereby preventing its further interaction with the relevant G-protein. This is followed by a clathrin-mediated internalization or 'sequestration' of the mAChR away from the plasma membrane and into an intracellular (endosomal) environment. Prolonged agonist activation of the receptor leads to the downregulation and proteolytic cleavage of the mAChR, a process that occurs in the lysosomes.

Muscarinic receptor subtypes are not uniformly distributed throughout the CNS and are present at different subcellular locations

Immunocytochemical and immunoprecipitation studies have revealed that the M1 subtype is the predominant form of the mAChR in the CNS and is found at postsynaptic locations (principally on the dendrites) in the cerebral cortex, hippocampus, striatum and thalamus. M2 mAChRs are enriched in the brainstem and thalamus, but also are found in the cerebral cortex, hippocampus and striatum. Many of the M2 receptors are localized to cholinergic nerve terminals where they serve as autoreceptors and, when activated, negatively regulate the release of ACh. M3 and M5 mAChRs are expressed at much lower densities than either the M1 or M2 subtypes. However, M3 receptors have been identified in the cerebral cortex and hippocampus, whereas the M5 subtype appears to be highly localized to the ventral tegmental area/substantia nigra region of the brain. Although M4 mAChRs are found in many brain regions, they are most plentiful in the striatum, where they regulate dopamine release. Also, like the M2 subtype, the M4 receptor can serve as an autoreceptor on cholinergic nerve terminals.

Muscarinic receptors in the CNS have been implicated in a number of neuropsychiatric disorders

mAChRs regulate a large number of central functions in the CNS including cognitive, behavioral, sensory, motor and reward processes. For example, it has long been known that either the administration of muscarinic antagonists or the lesioning of the cholinergic projections in the basal forebrain leads to cognitive impairment. These observations have led to the suggestion that mAChRs, in particular the M1 receptor that is the predominant subtype present in the higher brain areas involved in cognitive processes (cerebral cortex and hippocampus), may play a role in the etiology of Alzheimer's disease (AD). This possibility is supported by the clinical use of AChE inhibitors such as donepezil, which, via an increase in the availability of synaptic ACh, would increase the likelihood of activation of postsynaptic M1 receptors. Furthermore, M1 receptor signaling may inhibit the deposition of Aβ, a prominent pathological feature of AD (Chap. 46). Although the total number of M1 mAChRs in AD brain is the same as in age-matched controls, the proportion of the receptors that exist in the high affinity conformation state for agonists is reduced—a result that might indicate a reduction in the capacity of mAChR signaling in AD brain.

In schizophrenia, mAChR density (M1 and M4 subtypes) in postmortem brain is reduced in the prefrontal cortex, hippocampus and striatum when compared to control subjects (see Chap. 58). Imaging studies also indicate a significant reduction in the mAChR density in the cerebral cortex and basal ganglia of unmedicated schizophrenic patients. Furthermore, a single nucleotide polymorphism in the M1 mAChR gene (C267A) is associated with prefrontal cortical dysfunction in schizophrenic patients (Liao et al., 2003; Langmead et al., 2008). In keeping with the possibility of a deficit in mAChR signaling, AChE inhibitors can reduce the number of manic episodes and thought disturbances in schizophrenia.

The hallmark of Parkinson's disease is a reduction in the number of dopaminergic neurons in the *substantia nigra* and the loss of proper balance between striatal dopamine and cholinergic neurotransmission required for coordinated locomotor control. The resulting "cholinergic dominance" is ameliorated clinically by the administration of mAChR antagonists such as benztropine or trihexyphenidyl.

mAChRs have also been implicated in drug dependence, since the M5 subtype displays a very discrete CNS localization to the ventral tegmental area (VTA), a brain region closely associated with the reward pathway (see Chap. 61). In keeping with a putative role for mAChRs in drug addiction, intra-VTA administration of mAChR antagonists reduces dopamine release in the nucleus accumbens (Forster et al., 2001).

Transgenic mice permit an assessment of the physiological roles of individual subtypes *in vivo*

Until recently, an attribution of physiological roles for individual subtypes within the CNS has been hampered by the lack of pharmacological agents that exhibit the required degree of selectivity and the fact that most tissues possess multiple mAChR subtypes, which may act in a concerted fashion. To circumvent these problems, several laboratories have now generated transgenic mice that are deficient in one or more of the mAChR subtypes. Such animals are viable, fertile and display no major abnormal morphological characteristics. The first transgenic mouse to be generated was the M1 knockout (k/o), largely because of the interest in the role that this subtype may play in learning and memory. Unexpectedly, the initial behavioral analysis of M1 k/o mice indicated that hippocampus-dependent learning was relatively intact, as determined by Morris water maze and contextual fear-conditioning tests. However, subsequent tests demonstrated that the M1 k/o mouse was significantly impaired in tasks involving non–matching-to-sample working memory as well as consolidation. These results could indicate that the M1 receptor is not involved in the initial stability of memory but is required for memory processes involving an interaction between the cerebral cortex and hippocampus (Anagnostaras et al., 2003). Activation of downstream effectors such as phosphoinositide turnover and MAPK activation are essentially abolished in primary cortical neurons and in CA1 hippocampal pyramidal neurons obtained from M1 k/o mice. Since MAPK activity is considered to be a key factor in cognition and synaptic plasticity (Adams & Sweatt, 2002), these biochemical observations also support a role for the M1 receptor in learning and memory (see further discussion in Chap. 56). A more dramatic difference between control and M1 k/o

animals is seen in response to systemic pilocarpine. In control animals, the administration of pilocarpine induces seizures, and this model is used as an experimental paradigm for epilepsy. However, the M1 k/o mouse is completely resistant to the muscarinic agonist, indicating a central role for the M1 subtype in this model of epilepsy.

M2 k/o mAChR mice demonstrate significant deficits in the passive avoidance test, working memory, and both short- and long-term potentiation in the hippocampus, results that indicate an important role in learning and memory for this subtype (Seeger et al., 2004). Approximately 80–90% of the mAChRs in the spinal cord are of the M2 subtype (Duttaroy et al., 2002). Thus, not unexpectedly, M2 k/o mice display a profound reduction, although not a complete loss, of muscarinic agonist–induced analgesia. However the analgesic effect of muscarinic agonists is abolished in the double k/o M2/M4 mice. The M2 receptor also plays a role in the maintenance of body temperature since the ability of muscarinic agonists to induce hypothermia is reduced in the M2 k/o mouse. *In vitro* studies indicate that the autoreceptor function of M2 and M4 receptors is also lost from cholinergic neurons in the hippocampus and striatum in k/o mice.

In keeping with the concept that many cholinergic and dopaminergic pathways are functionally and anatomically connected, M4 k/o mice display an increase in basal locomotor activity, an effect that is facilitated by the administration of a D1 receptor agonist. Both M4 mAChRs and dopamine D1 receptors are localized postsynaptically on a population of striatal GABAergic projection neurons, the stimulation of which leads to the activation of a striatonigral pathway and the facilitation of locomotion. Activation of the M4 mAChRs serves to dampen the D1 dopamine receptor signaling. In addition, there is evidence that M4 mAChRs may regulate the activity of midbrain dopaminergic neurons involved with the reward pathway, since an enhanced basal- and amphetamine-stimulated efflux of dopamine is observed in the nucleus accumbens of M4 k/o mice. One possibility is that the M4 mAChRs are autoreceptors present on nerve terminals derived from cholinergic neurons that project from the laterodorsal tegmental nuclei and pedunculopontine nuclei and terminate in the VTA. Activation of the M4 receptors is hypothesized to inhibit ACh release and to limit the stimulatory effect of ACh on dopaminergic neurons in the VTA. M4 mAChRs may also play a role in the maintenance of pre-pulse inhibition (PPI) of the startle reflex (see Chap. 58), since M4 k/o mice display a significant increase in sensitivity to the PPI-disrupting effects of phencyclidine. Thus M4 mAChRs may play a role in CNS diseases such as schizophrenia in which hyperdopaminergic signaling occurs (see further discussion in Chap. 58).

Less is known about the function of the M3 and M5 mAChRs in the CNS, and both are relatively minor constituents of the pool of CNS mAChRs. However, M3 k/o mice are hypophagic and lean, an effect that may result from an altered hypothalamic appetite regulation. The most characteristic feature of M5 k/o mice is that they display a significant reduction in the rewarding effects of morphine, an effect that is accompanied by a reduction in morphine-stimulated dopamine efflux from the nucleus accumbens. Activation of M5 mAChRs is thought to increase the activity of the dopaminergic neurons in the VTA that project to the nucleus accumbens. The M5 mAChR is also present on the endothelial cells of brain blood vessels, where they mediate cholinergic relaxation.

Pharmacological therapies are used to treat cholinergic disorders

AChE inhibitors and muscarinic cholinergic agonists and antagonists have been used therapeutically to treat cholinergic insufficiency or overactivity. For example in the CNS, AChE inhibitors such as donepezil, which are presumed to increase the availability of ACh and thereby result in a greater activation of mAChRs, are currently prescribed for the treatment of AD. Muscarinic antagonists such as benztropine and trihexyphenidyl are used to reduce the cholinergic dominance found in Parkinson's disease. The symptomatic effects of these drugs in the CNS are modest. In the PNS, inhibitors of AChE are used to treat glaucoma and myasthenia gravis. Muscarinic agonists are also used to restore a loss of function; for example bethanechol can remedy a loss of gastric or urinary tone, whereas pilocarpine is used to treat xerostomia (an autoimmune malfunction of lacrimal and salivary glands). Muscarinic antagonists are also clinically useful. For example, pirenzepine has been used in the past to reduce gastric secretion in order to treat peptic or duodenal ulcers but the high doses needed also produced dry mouth and blurred vision. Currently, proton pump inhibitors and H2 histamine antagonists are the current drugs of choice for reducing gastric acidity. Atropine and scopolamine have been used to treat irritable bowel syndrome, and tolteridone can ameliorate an overactive bladder. mAChR antagonists such as scopolamine are also used to treat vertigo.

Given the abundance of mAChRs in the CNS and their role in neuropsychiatric disorders, the possibility of regulating the activity of the individual subtypes by means of pharmacologically selective agonists and antagonists has received considerable interest. However, to date these attempts have been largely unsuccessful, principally due to difficulties encountered in identifying agents that possess the required degree of subtype specificity. A major obstacle has been that the amino acid sequence at the agonist-/antagonist- (orthosteric) binding site is highly conserved among the five subtypes. In contrast, allosteric sites on mAChR subtypes have recently been discovered that are less well conserved and are located on the extracellular domains of the mAChR. As is the case for allosteric sites on nAChRs, as discussed above, subtype-selective ligands that act at these allosteric mAChR sites have been identified, and most do not activate the receptor in the absence of the orthosteric ligand. These studies suggest that the use of allosteric ligands could represent a potentially useful means to selectively regulate the activities of M1–M5 subtypes (Conn et al., 2009).

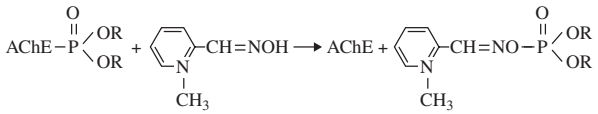
INHIBITION OF AChE: A TERRORIST TARGET AND A POTENTIAL CONTRIBUTOR TO GULF WAR SYNDROME

Susan Wonnacott

On March 20th, 1995, a terrorist attack on the Tokyo subway involved the release of the nerve gas Sarin (isopropylmethylphosphonofluoridate) in five carriages on three subway lines (Okumura et al., 1996). Sarin is a potent inhibitor of AChE with an estimated lethal dose in humans of 1 mg. Like DFP, sarin forms an essentially irreversible phosphoryl–serine enzyme intermediate. In the subway attack, 12 people died and 5,000 were injured, with over 600 sufficiently injured to warrant hospital care. The most common symptoms were breathing difficulties, ocular pain, nausea and vomiting, and headaches, with death due to respiratory failure. These symptoms reflect the roles of the cholinergic system in the control of muscle contraction (notably the intercostal muscles and diaphragm used for breathing), and in parasympathetic and central nervous system functions (including the brainstem control of respiratory function, see Table 1). In severe cases red blood cell AChE activity was reduced by more than 80%, and in some cases took more than two months to return to normal levels.

BOX FIG. 13-1 **Emergency workers in the Tokyo attack.**

Treatment of victims, in the more affected cases, involved artificial ventilation to sustain breathing, and administration of atropine to inhibit muscarinic receptors, thereby reducing cholinergic transmission at parasympathetic and CNS synapses. Pyridine oximes, such as pralidoxime or PAM-2, have been developed as an antidote for organophosphorus poisoning (Jokanovic & Stojilikovic, 2006). These drugs are powerful nucleophiles that reactivate AChE by displacing the phosphoryl moiety from the catalytic serine to form a phosphorylated oxime, thus regenerating the serine residue.



Reactivation of phosphorylated AChE with pralidoxime and formation of reactivated enzyme and phosphorylated oxime.

A more insidious case of low-level poisoning has been suggested to contribute to 'Gulf War syndrome.' More than a

TABLE 1 **Signs and Symptoms of Poisoning with Organophosphorus Compounds**

Severity of Poisoning	Signs and Symptoms	Site of Action	Red Blood Cell AChE Activity (% of control)
Mild	Nausea, vomiting, diarrhea, salivation, sweating, bronchoconstriction, lachrymation, increased bronchial secretions, bradycardia	PNS muscarinic	>40%
	Headache, dizziness	Central	
Moderate	As above plus miosis (unreactivity to light), urinary/fecal incontinence	PNS muscarinic	20–40%
	Muscle fasciculations, tachycardia	Nicotinic	
	As above plus dysarthria, ataxia, mental confusion	Central	
Severe	As above	PNS muscarinic	<20%
	Plus muscle fasciculations in respiratory muscles	Nicotinic	
	As above plus coma, convulsions, tremor, bronchospasm, seizures, death due to respiratory depression	Central	

Adapted from Jokanovic et al., 2006.

INHIBITION OF ACHE: A TERRORIST TARGET AND A POTENTIAL CONTRIBUTOR TO GULF WAR SYNDROME (*cont'd*)

quarter of all U.S. veterans from the 1990–1991 Persian Gulf war report having a chronic illness with symptoms including fatigue; sleep disturbance; pain; cognitive and mood defects; and gastrointestinal, respiratory and skin problems. These processes are all influenced by central or peripheral cholinergic systems. Epidemiological studies have linked the incidence of symptoms with exposure to AChE inhibitors (Golomb, 2008), although this interpretation remains controversial.

Personnel deployed to the Persian Gulf in this period (about 700,000 in total) could have encountered these inhibitors in several ways:

BOX FIG. 13-3 Pyridostigmine, as given to US service personnel in the first Gulf War.

- An estimated 250,000 personnel received the carbamate AChE inhibitor pyridostigmine bromide as a protective agent against possible nerve agent exposure in the battlefield.
- Both carbamate and organophosphorus pesticides were used extensively to prevent insect-transmitted diseases.
- Demolition of munitions depots may have resulted in low-level organophosphorus exposure if they contained nerve agents.

References

Golomb, B. A. (2008). Acetylcholinesterase inhibitors and Gulf War illnesses. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 4295–4300.

Jokanovic, M., & Stojilikovic, M. P. (2006). Current understanding of the application of pyridinium oximes as cholinesterase reactivators in treatment of organophosphate poisoning. *European Journal of Pharmacology*, *553*, 10–17.

Okumura, T., Takasu, N., Ishimatsu, S., Miyanoki, S., Mitsuhashi, A., & Kumada, K., et al. (1996). Report on 640 victims of the Tokyo subway sarin attack. *Annals of Emergency Medicine*, *28*, 129–135.

References

Adams, J. P., & Sweatt, J. D. (2002). Molecular psychology: Roles for the ERK MAPK cascade in memory. *Annual Review of Pharmacology and Toxicology*, *42*, 135–163.

Anagnostaras, S. G., Murphy, G. G., Hamilton, S. E., Mitchell, S. L., Rhanama, N. P., Nathanson, N. M., et al. (2003). Selective cognitive dysfunction in acetylcholine M1 muscarinic mutant mice. *Nature Neuroscience*, *6*, 51–58.

Apparsundaram, S., Martinez, V., Parikh, V., Kozak, R., & Sarter, M. (2005). Increased capacity and density of choline transporters situated in synaptic membranes of the right medial prefrontal cortex of attentional task-performing rats. *Journal of Neuroscience*, *25*, 3851–3856.

Badio, B., & Daly, J. W. (1994). Epibatidine, a potent analgesic and nicotinic agonist. *Molecular Pharmacology*, *45*, 563–569.

Bazalakova, M. H., & Blakely, R. D. (2006). The high-affinity choline transporter: A critical protein for sustaining cholinergic signaling as revealed in studies of genetically altered mice. *Handbook of Experimental Pharmacology*, *175*, 525–544.

Bierut, L. J. (2010). Convergence of genetic findings for nicotine dependence and smoking related diseases with chromosome 15q24-25. *Trends in Pharmacological Sciences*, *31*, 46–51.

Bierut, L. J., Stitzel, J. A., Wang, J. C., Hinrichs, A. L., Grucza, R. A., Xuei, X., et al. (2008). Variants in nicotinic receptors and risk for nicotine dependence. *The American Journal of Psychiatry*, *165*, 1163–1171.

Birdsall, N. J. M., Burgen, A. S. V., & Hulme, E. C. (1978). The binding of agonists to brain muscarinic receptors. *Molecular Pharmacology*, *14*, 723–736.

Bordia, T., Campos, C., Huang, L., & Quik, M. (2008). Continuous and intermittent nicotine treatment reduces L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesias in a rat model of Parkinson's disease. *Journal of Pharmacology and Experimental Therapeutics*, *327*, 239–247.

Bowman, W. C. (2006). Neuromuscular block. *British Journal of Pharmacology*, *147*(Suppl. 1), S277–286.

Brejc, K., van Dijk, W. J., Klaassen, R. V., Schuurmans, M., van Der Oost, J., Smit, A. B., et al. (2001). Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature*, *411*, 269–276.

Celie, P. H., van Rossum-Fikkert, S. E., van Dijk, W. J., Brejc, K., Smit, A. B., & Sixma, T. K. (2004). Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron*, *41*, 907–914.

Champtiaux, N., & Changeux, J. P. (2004). Knockout and knockin mice to investigate the role of nicotinic receptors in the central nervous system. *Acetylcholine in the Cerebral Cortex*, *145*, 235–251.

Changeux, J. P. (2010). Nicotine addiction and nicotinic receptors: Lessons from genetically modified mice. *Nature Reviews Neuroscience*, *11*, 389–401.

Changeux, J. P., Devillers-Thiéry, A., & Chemouilli, P. (1984). Acetylcholine receptor: An allosteric protein. *Science*, *225*, 1335–1345.

Colquhoun, D., Ogden, D. C., & Mathie, A. (1987). Nicotinic acetylcholine receptors of nerve and muscle: Functional aspects. *Trends in Pharmacological Sciences*, *8*, 465–472.

Conn, P. J., Jones, C. K., & Lindsley, C. W. (2009). Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. *Trends in Pharmacological Sciences*, *30*, 148–155.

Cossins, J., Webster, R., Maxwell, S., Burke, G., Vincent, A., & Beeson, D. (2004). A mouse model of AChR deficiency syndrome with a phenotype reflecting the human condition. *Human Molecular Genetics*, *13*, 2947–2957.

Dale, H. H. (1914). The action of certain esters and ethers of choline, and their relation to muscarine. *Journal of Pharmacology and Experimental Therapeutics*, *6*, 147–190.

Dale, H. H., & Dudley, H. W. (1929). The presence of histamine and acetylcholine in the spleen of the ox and the horse. *Journal of Physiology*, *68*, 97–123.

Dale, H. H., Feldberg, W., & Vogt, Release of acetylcholine at voluntary motor nerve endings. *The Journal of Physiology (London)*, *86*, 353–380.

Dajas-Bailador, F., & Wonnacott, S. (2004). Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends in Pharmacological Sciences*, *25*, 317–324.

Duttaroy, A., Gomeza, J., Gan, J. W., Siddiqui, N., Basile, A. S., Harman, W. D., et al. (2002). Evaluation of muscarinic agonist-induced analgesia in muscarinic acetylcholine receptor knockout mice. *Molecular Pharmacology*, *62*, 1084–1093.

Ferguson, S. M., & Blakely, R. D. (2004). The choline transporter resurfaces: New roles for synaptic vesicles?. *Molecular Interventions*, *4*, 22–37.

Fisher, S. K. (1986). Inositol lipids and signal transduction at CNS muscarinic receptors. *Trends in Pharmacological Sciences Supplement: Subtypes of Muscarinic Receptors II*, 61–65.

Forster, G. L., Yeomans, J. S., Takeuchi, J., & Blaha, C. D. (2001). M5 muscarinic receptors are required for prolonged accumbal dopamine release after electrical stimulation of the pons in mice. *Journal of Neuroscience*, *21*(RC 190), 1–6.

Govind, A. P., Vezina, P., & Green, W. N. (2009). Nicotine-induced upregulation of nicotinic receptors: Underlying mechanisms and relevance to nicotine addiction. *Biochemical Pharmacology*, *78*, 756–765.

Grønlien, J. H., Håkerud, M., Ween, H., Thorin-Hagene, K., Briggs, C. A., Gopalakrishnan, M., et al. (2007). Distinct profiles of alpha7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. *Molecular Pharmacology*, *72*, 715–724.

Hasselmo, M. E., & Sarter, M. (2010). Modes and models of forebrain cholinergic neuromodulation of cognition. *Neuropsychopharmacology* (Epub ahead of print)

Johnson, G., Swart, C., & Moore, S. W. (2008). Interaction of acetylcholinesterase with the G4 domain of the laminin alpha1-chain. *Biochemical Journal*, *411*, 507–514.

Karlin, A. (2002). Emerging structure of the nicotinic acetylcholine receptors. *Nature Reviews Neuroscience*, *3*, 102–114.

Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., et al. (1986). Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature*, *323*, 411–416.

Kuryatov, A., Onksen, J., & Lindstrom, J. (2008). Roles of accessory subunits in alpha4beta2(*) nicotinic receptors. *Molecular Pharmacology*, *74*, 132–143.

Langley, J. N. (1906). On nerve endings and on special excitable substances in cells. *Proceedings of the Royal Society of London. Series B*, *78*, 170–194.

Langmead, C. J., Watson, J., & Reavill, C. (2008). Muscarinic acetylcholine receptors as CNS drug targets. *Pharmacology & Therapeutics*, *117*, 232–243.

Le Novère, N., & Changeux, J. P. (1995). Molecular evolution of the nicotinic acetylcholine receptor: An example of multigene family in excitable cells. *Journal of Molecular Evolution*, *40*, 155–172.

Leonard, S., Mexal, S., & Freedman, R. (2007). Smoking, genetics and schizophrenia: Evidence for self medication. *Journal of Dual Diagnosis*, *3*, 43–59.

Liao, D. L., Hong, C. J., Chen, Y. E., Lee, S. M., & Chang, C. Y. (2003). Association of muscarinic M1 receptor genetic polymorphisms with psychiatric symptoms and cognitive function in schizophrenic patients. *Neuropsychobiology*, *48*(2), 72–76.

Lindstrom, J. (2002). Autoimmune diseases involving nicotinic receptors. *Journal of Neurobiology*, *53*, 656–665.

Loewi, O. (1921). Ueber humorale Uebertragbarkeit der herznervenwirkung. *Pfluegers Archiv General Physiology*, *189*, 239–242.

Liu, Z., Zhang, J., & Berg, D. K. (2007). Role of endogenous nicotinic signaling in guiding neuronal development. *Biochemical Pharmacology*, *74*, 1112–1119.

Livingstone, P. D., & Wonnacott, S. (2009). Nicotinic acetylcholine receptors and the ascending dopamine pathways. *Biochemical Pharmacology*, *78*, 744–755.

Maehle, A. H., Prüll, C. R., & Halliwell, R. F. (2002). The emergence of the drug receptor theory. *Nature Reviews Drug Discovery*, *1*, 637–641.

Massoulié, J., & Millard, C. B. (2009). Cholinesterases and the basal lamina at vertebrate neuromuscular junctions. *Current Opinion in Pharmacology*, *9*, 316–325.

Massoulié, J. (2002). The origin of the molecular diversity and functional anchoring of cholinesterases. *Neurosignals*, *11*, 130–143.

McIntosh, J. M., Absalom, N., Chebib, M., Elgoyhen, A. B., & Vincler, M. (2009). Alpha9 nicotinic acetylcholine receptors and the treatment of pain. *Biochemical Pharmacology*, *78*, 693–702.

Millar, N. S., & Gotti, C. (2009). Diversity of vertebrate nicotinic acetylcholine receptors. *Neuropharmacology*, *56*, 237–246.

Murrin, L. C., & Kuhar, M. J. (1976). Activation of high affinity choline uptake *in vitro* by depolarizing agents. *Molecular Pharmacology*, *12*, 1082–1090.

Okuda, T., Haga, T., Kanai, Y., Endou, H., Ishihara, T., & Katsura, I. (2000). Identification and characterization of the high affinity choline transporter. *Nature Neuroscience*, *3*, 120–125.

Paton, W. D., & Zaimis, E. J. (1949). The pharmacological actions of polymethylene bistrimethylammonium salts. *British Journal of Pharmacology*, *120*(Suppl. 1997), 60–79.

Perrier, A. L., Massoulié, J., & Krejci, E. (2002). PRiMA: The membrane anchor of acetylcholinesterase in the brain. *Neuron*, *33*, 275–285.

Picciotto, M. R., Zoli, M., Rimondini, R., Léna, C., Marubio, L. M., Pich, E. M., et al. (1998). Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. *Nature*, *391*, 173–177.

Picciotto, M. R., Alreja, M., & Jentsch, D. (2002). Acetylcholine. In K. L. Davis, D. Charney, J. T. Coyle & C. Nemeroff (Eds.), *Neuropsychopharmacology: The fifth generation of progress* (pp. 1–14). Brentwood, TN: Amer. Coll. of Neuropsychopharmacology.

Quik, M., O'Neill, M., & Perez, X. A. (2007). Nicotine neuroprotection against nigrostriatal damage: Importance of the animal model. *Trends in Pharmacological Sciences*, *28*, 229–235.

Rollema, H., Coe, J. W., Chambers, L. K., Hurst, R. S., Stahl, S. M., & Williams, K. E. (2007). Rationale, pharmacology and clinical

efficacy of partial agonists of alpha4beta2 nACh receptors for smoking cessation. *Trends in Pharmacological Sciences*, *28*, 316–325.

Seeger, T., Fedorova, I., Zheng, F., Miyakawa, T., Koustova, E., Gomeza, J., et al. (2004). M2 muscarinic acetylcholine knock-out mice show deficits in behavioral flexibility, working memory and hippocampal plasticity. *Journal of Neuroscience*, *24*, 10117–10127.

Silman, I., & Sussman, J. L. (2005). Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. *Current Opinion in Pharmacology*, *5*, 293–302.

Silman, I., & Sussman, J. L. (2008). Acetylcholinesterase: How is structure related to function?. *Chemico-Biological Interactions*, *175*, 3–10.

Sine, S. M., & Engel, A. G. (2006). Recent advances in Cys-loop receptor structure and function. *Nature*, *440*, 448–455.

Soreq, H., & Seidman, S. (2001). Acetylcholinesterase—new roles for an old actor. *Nature Reviews Neuroscience*, *2*, 294–302.

Steinlein, O. K., & Bertrand, D. (2008). Neuronal nicotinic acetylcholine receptors: From the genetic analysis to neurological diseases. *Biochemical Pharmacology*, *76*, 1175–1183.

Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., et al. (1991). Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine-binding protein. *Science*, *253*, 872–879.

Sussman, J. L., & Silman, I. (1992). Acetylcholinesterase: Structure and use as a model for specific cation–protein interactions. *Current Opinion in Structural Biology*, *2*, 721–729.

Taylor, P., & Radic, Z. (1994). The cholinesterases: From genes to proteins. *Annual Review of Pharmacology and Toxicology*, *34*, 281–320.

Unwin, N. (2005). Refined structure of the nicotinic acetylcholine receptor at 4Å resolution. *Journal of Molecular Biology*, *346*, 967–989.

Verderio, C., Rossetto, O., Grumelli, C., Frassoni, C., Montecucco, C., & Matteoli, M. (2006). Entering neurons: Botulinum toxins and synaptic vesicle recycling. *Embo Journal*, *7*, 995–999.

Vetter, D. E., Katz, E., Maison, S. F., Taranda, J., Turcan, S., Ballestero, J., et al. (2007). The alpha10 nicotinic acetylcholine receptor subunit is required for normal synaptic function and integrity of the olivocochlear system. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 20594–20599.

Wess, J. (1996). Molecular biology of muscarinic acetylcholine receptors. *Crc Critical Reviews in Clinical Neurobiology*, *10*, 69–99.

Wess, J., Eglen, R. M., & Gautam, D. (2007). Muscarinic acetylcholine receptors: Mutant mice provide new insights for drug development. *Nature Reviews Drug Discovery*, *6*, 721–733.

Wessler, I., & Kirkpatrick, C. J. (2008). Acetylcholine beyond neurons: The non-neuronal cholinergic system in humans. *British Journal of Pharmacology*, *154*, 1558–1571.

Xu, W., Gelber, S., Orr-Urtreger, A., Armstrong, D., Lewis, R. A., Ou, C. N., et al. (1999). Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 5746–5751.

Xu, W., Orr-Urtreger, A., Nigro, F., Gelber, S., Sutcliffe, C. B., Armstrong, D., et al. (1999). Multiorgan autonomic dysfunction in mice lacking the beta2 and the beta4 subunits of neuronal nicotinic acetylcholine receptors. *Journal of Neuroscience*, *19*, 9298–9305.